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14. ABSTRACT
Our recent work has demonstrated that: 1) 15-lipoxygenase 2 (15-LOX2), which metabolizes AA to generate 15(S)-HETE, is the major LOX expressed in adult prostate epithelial cells but down-regulated or lost in PCa in vitro as wellas in vivo; 2) 15-LOX2 expression is inversely correlated with the pathological grade and Gleason scores of PCapatients; 3) 15-LOX2 is a negative cell-cycle regulator in normal human prostate (NHP) epithelial cells; 4) 15(S)-HETE inhibits PCa cell migration and invasion and re-expression of 15-LOX2, or its splice variant 15-LOX2sv-b,inhibits PCa cell proliferation in vitro and tumor development in vivo; 5) 15-LOX2 expression in NHP cells ispositively regulated by the transcription factor Sp1 and negatively regulated by Sp3; 6) The expression of 15-LOX2and its multiple splice variants in NHP cells is cell-autonomously induced and is correlated with and causally involvedin the senescence of NHP cells; 7) Transgenic expression of 15-LOX2, or its splice variant, 15-LOX2sv-b, in mouseprostate induces unique gene expression profile and causes 'degenerative' prostate overgrowth; and 8) Finally, 15-LOX2 transgene expression inhibits TRAMP tumor development in the compound mice. These observations togethersupport our initial hypothesis that 15-LOX2 represents a functional prostate tumor suppressor, whose loss ofexpression contributes to PCa development. We initially proposed two Specific Aims: 1) to test the hypothesis that15-LOX2 inhibits PCa development in an orthotopic implantation model using an inducible 15-LOX2 expressionsystem; and 2) to test the hypothesis that 15-LOX2 inhibits PCa development in newly developed prostate-specifictransgenic animal models. We have now accomplished both Specific Aims (see detailed Progress Report).

15. SUBJECT TERMS
Prostate cancer, apoptosis, Bcl-2, Bim

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Table of Contents

INTRODUCTION

Clinical, laboratory, and epidemiological studies have provided strong evidence that bioactive lipids can modulate prostate cancer (PCa) susceptibility, development, and progression. Studies from our lab and others' have implicated one class of lipid molecules, arachidonic acid (AA) lipoxygenases (LOXs) and their products, in prostate tumorigenesis. AA released from the membrane phospholipid is metabolized by several LOXs to various fatty acid molecules that play signaling functions. Our recent work has implicated one LOX molecule, i.e., 15-LOX2, in prostate cancer. Specifically, our large body of observations suggests that *15-LOX2 may represent a functional prostate tumor suppressor, whose loss of expression contributes to PCa development*. Our working hypothesis is that 15-LOX2 is an endogenous PCa suppressor. Our objective is to test whether restoration/overexpression of 15-LOX2 expression could inhibit PCa development *in vivo*.

BODY (the grant period: April 1, 2003 – March 31, 2006)

In our Statement of Work (SOW), we proposed the following two Tasks.

Task 1. To test the hypothesis that 15-LOX2 inhibits PCa development in an orthotopic implantation model using an inducible 15-LOX2 expression system (Months 1-18)
  a. To assess the impact of 15-LOX2 expression on PCa development using an orthotopic implantation model (Months 1-12). Approximately 300 SCID mice will be needed.
  b. To determine the molecular mechanisms of 15-LOX2-regulated PCa development (Months 10-18).

Task 2. To test the hypothesis that 15-LOX2 inhibits PCa development in newly developed prostate-specific transgenic animal models
  a. To characterize the phenotypes of transgenic prostates overexpressing 15-LOX2 or 15-LOX2sv-b (Months 1-8). A total of ~ 300 founder mice and F_1 offspring will be used for this phase of work.
  b. To cross the PB-15LOX2 transgenic animals to TRAMP mice and analyze PCa development in the resultant offspring (Months 8-36). A total of ~1,450 PB-15LOX$_2$ transgenic mice and TRAMP mice will be needed for this work.

We have completed the work proposed in Task 1, with four manuscripts published (***Append. I-IV***; attached). The first manuscript (*J. Biol. Chem. 278: 25091-25100, 2003*) addresses Task 1a, i.e., to demonstrate the tumor-suppressive functions of restoration of 15-LOX2 expression in PCa cells. More importantly, this paper also partly elucidates the molecular mechanisms of 15-LOX2 functions in showing that 15-LOX2 is a nuclear protein and that the tumor-suppressive functions of 15-LOX2 does not absolutely require its AA-metabolizing activity. The Abstract of this paper is as follows.

"15-Lipoxygenase 2 (15-LOX2), the most abundant arachidonate (AA)-metabolizing enzyme expressed in adult human prostate, is a negative cell-cycle regulator in normal human prostate epithelial cells (J. Biol. Chem. 277: 16189-16201, 2002). Here we study the subcellular distribution of 15-LOX2 and report its tumor-suppressive functions. Immunocytochemistry and biochemical fractionation reveal that 15-LOX2 is expressed at multiple subcellular locations including cytoplasm, cytoskeleton, cell-cell border as well as nucleus. Surprisingly, the three splice variants of 15-LOX2 we previously cloned, i.e., 15-LOX2sv-a/b/c, are mostly excluded from the nucleus. A potential bi-partite nuclear localization signal (NLS), R^{203}KGLWRSLNEMKRIFNFRR^{221}, is identified in the N-terminus of 15-LOX2, which is retained in all splice variants. Site-directed mutagenesis reveals that this putative NLS is only partially involved in the nuclear import of 15-LOX2. To elucidate the relationship between nuclear localization, enzymatic activity, and tumor suppressive functions, we established PCa cell clones stably expressing 15-LOX2 or 15-LOX2sv-b. The 15-LOX2 clones express 15-LOX2 in the nuclei and possess robust

enzymatic activity whereas 15-LOX2sv-b clones show neither nuclear protein localization nor AA-metabolizing activity. To our surprise, both 15-LOX2 and 15-LOX2sv-b stable clones proliferate much slower in vitro when compared to control clones. More importantly, when orthotopically implanted in nude mouse prostate, both 15-LOX2 and 15-LOX2sv-b suppress PC3 tumor growth in vivo. Together, these results suggest that both 15-LOX2 and 15-LOX2sv-b suppress prostate tumor development and the tumor-suppressive functions apparently do not necessarily depend on AA-metabolizing activity and nuclear localization."

In the second paper published (*Oncogene 23: 6942-6953, 2004*), we addressed the potential mechanisms of action of 15-LOX2 (i.e., part of Task 1b) and its PCa-suppressive functions from a different angle. Specifically, we attempted to address what could be responsible for the silencing of 15-LOX2 expression in PCa cells in vitro and in vivo. As a first step, we attempted to elucidate how 15-LOX2 expression in normal human prostate epithelial cells is regulated at the molecular level. Our results reveal that the transcription factors Sp1 and Sp3 play critical positive and negative roles, respectively, in regulating 15-LOX2 gene expression. By contrast, AR does not appear to directly regulate 15-LOX2 gene expression. The Abstract of this paper is attached below.

"15-Lipoxygenase 2 (15-LOX2), the most abundant arachidonate-metabolizing LOX in adult human prostate, is a negative cell-cycle regulator in normal human prostate (NHP) epithelial cells (J. Biol. Chem. 277: 16189-16201, 2002). This project was undertaken to address how basal-level 15-LOX2 expression is regulated in NHP cells. Through detailed in silico promoter examination and promoter deletion and activity analysis, we found that several Sp1 sites (i.e., 3 GC boxes and 1 CACCC box) in the proximal promoter region play a critical role in regulating 15-LOX2 expression in NHP cells. Several pieces of evidence further suggest that the Sp1 and Sp3 proteins play a physiologically important role in positively and negatively regulating the 15-LOX2 gene expression, respectively. First, mutations in the GC boxes affected the 15-LOX2 promoter activity. Second, both Sp1 and Sp3 proteins were detected in the protein complexes that bound the GC boxes revealed by EMSA. Third, importantly, inhibition of Sp1 activity or overexpression of Sp3 both inhibited the endogenous 15-LOX2 mRNA expression. Since 15-LOX2 is normally expressed in the prostate luminal epithelial cells, we subsequently explored whether androgen/androgen receptor (AR) may directly regulate its gene expression. The results indicate that androgen does not directly regulate 15-LOX2 gene expression. Together, these observations provide insight on how 15-LOX2 gene expression may be regulated in NHP cells. Future work will focus on how 15-LOX2 expression in suppressed in prostate cancer cells."

The third paper (*Oncogene, 24: 3583-3595, 2005*) further explores the molecular mechanisms of action of 15-LOX2 (Task 1b) but the results have great impact on the goal of Task 2a. It provides direct evidence linking 15-LOX2 to the replicative senescence of normal human prostate epithelial cells. As all senescence genes are known to be tumor suppressors, these results shed light on how 15-LOX2 might be functioning as prostate tumor suppressors. Importantly, as we shall discuss below, the 15-LOX2 transgenic mice show a premature senescence phenotype in prostate. The Abstract of this paper is presented below.

"Normal human prostatic (NHP) epithelial cells undergo senescence in vitro and in vivo, but little is known about the tissue-specific molecular mechanisms. Here we first characterize young primary NHP cells as CK5$^+$/CK18$^+$ intermediate basal cells that also express several other putative stem/progenitor cell markers including p63, CD44, α2β1, and hTERT. When cultured in serum and androgen-free medium, NHP cells gradually lose the expression of these markers, slow down in proliferation, and enter senescence. Several pieces of evidence implicate 15-lipoxygenase 2 (15-LOX2), a molecule with a restricted tissue expression and most abundantly expressed in adult human prostate, in the replicative senescence of NHP cells. First, the 15-LOX2 promoter activity and the mRNA and protein levels of 15-LOX2 and its multiple splice variants are up-regulated in serially passaged NHP cells, which precede replicative senescence and occur in a cell-autonomous manner. Second, all immortalized prostate epithelial cells and prostate cancer (PCa) cells do not express 15-LOX2. Third,

PCa cells stably transfected with 15-LOX2 or 15-LOX2sv-b, a splice variant that does not possess arachidonate-metabolizing activity, show a passage-related senescence-like phenotype. Fourth, infection of early-passage NHP cells with retroviral vectors encoding 15-LOX2 or 15-LOX2sv-b induces partial cell-cycle arrest and big and flat, senescence-like phenotype. Finally, 15-LOX2 protein expression in human prostate correlates with age. Together, these data suggest that 15-LOX2 may represent an endogenous prostate senescence gene and its tumor-suppressing functions might be associated with its ability to induce cell senescence."

The fourth paper is a recent Review paper that summarizes our current knowledge on the potential roles and mechanisms of action of 15-LOX2 in NHP cells and prostate cancer.

For Task 2a, we have established several transgenic founder lines for both 15-LOX2 and 15-LOX2sv-b and have thoroughly characterized the transgene expression. Remarkably, we have observed that transgenic prostate shows 'degenerative' changes suggestive of premature senescence associated with NHP cell overgrowth (i.e., uncontrolled size increase) and 'super-secretion'. We have carried out systematic proteomic and microarray analyses to characterize the impact of transgene (i.e., 15-LOX2 or 15-LOX2sv-b) expression on mouse prostate development and the results are being summarized into a high-impact journal article. In the mean time, we have also crossed these transgenic lines with the TRAMP lines to determine whether 15-LOX2 expression suppresses PCa development in the TRAMP model (i.e., Task 2b). This is a very lengthy and tedious, collaborative project with Dr. Russell Klein in Ohio State university. The overall experiments have been accomplished although the completion of analyzing all data will likely take another 6-10 months (several hundreds of animals). Our preliminary results suggest that, as expected, 15-LOX2 transgene expression partially inhibits PCa development in TRAMP mice. This last part of the work will also be summarized into a separate manuscript.

KEY RESEARCH ACCOMPLSIMENTS

--- 15-LOX2 has multiple splice variants, most of which lack AA-metabolizing activities.

---15-LOX2 is localized in multiple subcellular compartments with especially prominent distribution in the nucleus but its splice variants are largely excluded from the nucleus.

--- Re-expression of 15-LOX2 in PCa cells, which do not express endogenous 15-LOX2, inhibits tumor cell proliferation in vitro and tumor development in vivo in an orthotopic implantation model.

--- Surprisingly, expression of 15-LOX2sv-b, which does not localize to the nucleus and does not metabolize AA to produce 15(S)-HETE, also inhibits tumor cell proliferation in vitro and retards tumor development in vivo, suggesting that the tumor-suppressive functions of 15-LOX2 do not necessarily require its nuclear targeting or AA-metabolizing activity.

--- The 15-LOX2 gene expression in normal human prostate epithelial cells is regulated positively and negatively, respectively, by the Sp1 and Sp3 transcription factors. These results suggest that the silencing of 15-LOX2 expression in PCa cells might be related to abnormal Sp1/Sp3 signaling functions.

--- Expression of 15-LOX2 and its multiple splice variants is cell-autonomously induced in cultured NHP cells, is linked to and causally involved in the replicative senescence of normal NHP cells, suggesting that the prostate tumor-suppressive functions of 15-LOX2 may be related to its functions to induce cell senescence.

--- We have successfully targeted 15-LOX2 and 15-LOX2sv-b to the mouse prostate in transgenic animals. The transgene, as expected, is most strongly expressed in the ventral prostate (VP), followed by, in descending orders, lateral (LP), dorsal (DP), and anterior (AP) prostates. Remarkably, the transgenic prostates show some premature senescence phenotype associated with deregulated epithelial overgrowth and 'super-secretion'.

--- We are also crossed the 15-LOX2 or 15-LOX2sv-b transgenic animals with the TRAMP mice to examine their effects on tumor development in TRAMP models. The preliminary results suggest that transgenic expression of 15-LOX2 or 15-LOX2sv-b also inhibits TRAMP tumor development.

REPORTABLE OUTCOMES

Bhatia, B., Maldonado, C., Tang, S-H., Chandra, D., Klein, R.D., Chopra, D., Shappell, S., Yang, P., Newman, R.A., and **Tang, D.G**. Subcellular localization and tumor-suppressive functions of 15-lipoxygenase 2 (15-LOX2) and its splice variants. J. Biol. Chem. 278, 25091-25100, 2003.

Tang S, Bhatia B, Zhou J, Maldonado CJ, Chandra D, Kim E, Fischer SM, Butler AP, Friedman SL, **Tang DG.** Evidence that Sp1 positively and Sp3 negatively regulate and androgen does not directly regulate functional tumor suppressor 15-lipoxygenase 2 (15-LOX2) gene expression in normal human prostate epithelial cells. Oncogene 23: 6942-6953, 2004.

Bhatia B, Tang S, Yang P, Doll A, Aumüeller G, Newman RA, **Tang DG.** Cell-autonomous induction of functional tumor suppressor 15-lipoxygenase 2 (15-LOX2) contributes to replicative senescence of human prostate progenitor cells. Oncogene, 24: 3583-3595, 2005.

Tang, D.G., Bhatia B, Tang S, and Schneider-Broussard, R. 15-lipoxygenase 2 (15-LOX2) is a functional prostate tumor suppressor that regulates prostate epithelial cell growth (i.e., size), differentiation, and senescence. *Prostaglandins & Other Lipid Mediators* 2006, *in press.*

In addition to the above-referenced manuscripts directly related to the 15-LOX2 project, the following publications, which are also related to prostate cancer development, have also obtained indirect support for the current DOD Idea Award.

Chandra, D., Choy, G., Deng, X., Bhatia, B., and Tang, D.G. Active caspase-8 on the outer mitochondrial membrane cleaves BAP31 and caspase-3 and mediates mitochondria – ER crosstalk in etoposide-induced apoptosis. *Mol. Cell. Biol.* 24: 6592-6607, 2004.

Liu, J-W., Chandra, D., Rudd, M.D., Butler, A.P., Pallotta, V., Brown, D., Coffer, P.J., and Tang, D.G. Induction of pro-survival molecules by apoptotic stimuli: Involvement of FOXO3a and ROS. *Oncogene* 24: 2020-2031, 2005.

Chandra, D., Choy, G., Daniel, P.T., and Tang, D.G. Bax-dependent regulation of Bak by voltage-dependent anion channel 2. *J. Biol. Chem.* 280: 19051-19061, 2005.

Patrawala, L., Calhoun, T., Schneider-Broussard, R., Zhou, J., Claypool, K., and Tang, D.G. Side population (SP) is enriched in tumorigenic, stem-like cancer cells whereas ABCG2^{+} and ABCG2^{-} cancer cells are similarly tumorigenic. *Cancer Res*. 65: 6207-6219, 2005.

Patrawala L, Calhoun T, Schneider-Broussard R, Li H, Bhatia B, Tang S, Reilly JG, Chandra D, Zhou J, Claypool K, Coghlan L, and Tang DG. Highly purified CD44^{+} prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. *Oncogene* 25, 1696-1708, 2006.

Tang, D.G., Patrawala L, Calhoun T, Bhatia B, Schneider-Broussard R, Choy, G., and Jeter, C. Prostate cancer stem/progenitor cells: Identification, characterization, and implications. *Mol. Carcinog.*, 2006, *in press*.

Chandra, D., Bratton, S.B., Person, M.D., Tian, Y., Martin, A., Ayers, M., Fearnhead, H.O., Gandhi, V., and Tang, D.G. Intracellular nucleotides act as critical prosurvival factors by binding to cytochrome C and inhibiting formation of Apaf-1 apoptosome. Cell 2006, *in revision*.

Patrawala, L., Bhatia, B., Calhoun, T., Schneider-Broussard, R., and Tang, D.G. Loss of progenitor markers accompanies the senescence of normal human prostate (NHP) progenitor cells and enhancement of tumorigenicity by senescent NHP cells. ***Oncogene*** 2006, *in press*.

Bhatia, B., Multani, A.S., Patrawala, L., Calhoun, T., Zhou, J., Schroeder, L., Schneider-Broussard, R., Shen, J-J., Pathak, S., Chang, S., and Tang, D.G. Evidence that cell fusion with presenescent/senescent human prostate epithelial cells enhances tumorigenicity: Inhibition by p16 and hTERT. ***Oncogene*** 2006, *in press*.

Choy, G., Chandra, D., and Tang, D.G. Mitochondrial release or de novo transcriptional activation mediates distinct pro-death or pro-survival functions of HSP60. ***J. Biol. Chem***. 2006, *in revision*.

Li, H-W., Calhoun, T., Schneider-Broussard, R., Patrawala, L., Choy, G., Jiang, M., V. Speights, V., Waxman, J., Hayward, S., and Tang, D.G. Crucial role of microenvironment in reconstituting both tumorigenic and metastatic potentials of human prostate cancer cells in mice: Evidence that metastatic cells represent a more rare and primitive subset of tumor-reinitiating cells. 2006, *submitted*.

Bhatia, B., Jiang, M., Patrawala, L., Schneider-Broussard, R, Calhoun T., Multani, A., Chang, S., Hayward, S, and Tang, D.G. Genetically defined immortalization of normal human prostate (NHP) epithelial progenitor cells with tri-potential differentiating ability *in vivo*. 2006, *submitted*.

CONCLUSIONS

We have accomplished the goals proposed in the SOW. There is no doubt that 15-LOX2 is an important and powerful prostate tumor suppressor. Our recent results suggest that 15-LOX2 suppresses PCa development possibly by functioning as an endogenous senescence gene.

REFERENCES

N/A.

APPENDICES

Append. I. Bhatia, B., Maldonado, C., Tang, S-H., Chandra, D., Klein, R.D., Chopra, D., Shappell, S., Yang, P., Newman, R.A., and Tang, D.G. Subcellular localization and tumor-suppressive functions of 15-lipoxygenase 2 (15-LOX2) and its splice variants. *J. Biol. Chem*. 278, 25091-25100, 2003.

Append. II. Tang S, Bhatia B, Zhou J, Maldonado CJ, Chandra D, Kim E, Fischer SM, Butler AP, Friedman SL, Tang DG. Evidence that Sp1 positively and Sp3 negatively regulate and androgen does not directly regulate functional tumor suppressor 15-lipoxygenase 2 (15-LOX2) gene expression in normal human prostate epithelial cells. *Oncogene* 23: 6942-6953, 2004.

Append. III. Bhatia B, Tang S, Yang P, Doll A, Aumüeller G, Newman RA, Tang DG. Cell-autonomous induction of functional tumor suppressor 15-lipoxygenase 2 (15-LOX2) contributes to replicative senescence of human prostate progenitor cells. *Oncogene* 24: 3583-3595, 2005.

Append. IV. Tang, D.G., Bhatia B, Tang S, and Schneider-Broussard, R. 15-lipoxygenase 2 (15-LOX2) is a functional prostate tumor suppressor that regulates prostate epithelial cell growth (i.e., size), differentiation, and senescence. *Prostaglandins & Other Lipid Mediators* 2006, *in press.*

Subcellular Localization and Tumor-suppressive Functions of 15-Lipoxygenase 2 (15-LOX2) and Its Splice Variants*

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15-Lipoxygenase 2 (15-LOX2), the most abundant arachidonate (AA)-metabolizing enzyme expressed in adult human prostate, is a negative cell-cycle regulator in normal human prostate epithelial cells. Here we study the subcellular distribution of 15-LOX2 and report its tumor-suppressive functions. Immunocytochemistry and biochemical fractionation reveal that 15-LOX2 is expressed at multiple subcellular locations, including cytoplasm, cytoskeleton, cell-cell border, and nucleus. Surprisingly, the three splice variants of 15-LOX2 we previously cloned, *i.e.* 15-LOX2sv-a/b/c, are mostly excluded from the nucleus. A potential bi-partite nuclear localization signal (NLS), [203]RKGLWRSLNEMKRIFN-FRR[221], is identified in the N terminus of 15-LOX2, which is retained in all splice variants. Site-directed mutagenesis reveals that this putative NLS is only partially involved in the nuclear import of 15-LOX2. To elucidate the relationship between nuclear localization, enzymatic activity, and tumor suppressor functions, we established PCa cell clones stably expressing 15-LOX2 or 15-LOX2sv-b. The 15-LOX2 clones express 15-LOX2 in the nuclei and possess robust enzymatic activity, whereas 15-LOX2sv-b clones show neither nuclear protein localization nor AA-metabolizing activity. To our surprise, both 15-LOX2- and 15-LOX2sv-b-stable clones proliferate much slower *in vitro* when compared with control clones. More importantly, when orthotopically implanted in nude mouse prostate, both 15-LOX2 and 15-LOX2sv-b suppress PC3 tumor growth *in vivo*. Together, these results suggest that both 15-LOX2 and 15-LOX2sv-b suppress prostate tumor development, and the tumor-suppressive functions apparently do not necessarily depend on AA-metabolizing activity and nuclear localization.

15-Lipoxygenase 2 (15-LOX2)[1] is a recently cloned lipoxygenase that shows the highest homology (~80% amino acid identity) to murine 8-LOX, with ~40% identity to human 5-LOX, 12-LOX, or 15-LOX1 (1). It has at least three splice variants (termed 15-LOX2sv-a/b/c) (2, 3) and metabolizes preferentially arachidonic acid (AA) to 15(*S*)-hydroxyeicosatetraenoic acid (15(*S*)-HETE) (1). 15-LOX2 shows an interesting tissue expression pattern, *i.e.* mainly in prostate, lung, skin, and cornea (1–3). This tissue-restricted expression pattern suggests that 15-LOX2 may play a role in the normal development and its abnormal expression/function may contribute to tumorigenesis in these organs. Indeed, work by Shappell *et al.* (4–6) indicates that 15-LOX2 mRNA, protein expression, and enzymatic activity are decreased in high grade prostate intraepithelial neoplasia (PIN) and prostate cancer (PCa), and the expression levels of 15-LOX2 are inversely correlated with the pathological grade (Gleason scores) of the patients. We recently reported that 15-LOX2 is a negative cell-cycle regulator in normal human prostate (NHP) epithelial cells (3). These observations (3–6) together raise the possibility that 15-LOX2 may represent an endogenous prostate tumor suppressor, and its down-regulation may contribute to PCa development. Here we provide experimental data in support of this possibility as restoration of 15-LOX2 expression inhibits PCa cell proliferation *in vitro* and tumor development *in vivo*. We further show that the tumor-suppressive functions of 15-LOX2 do not necessarily depend on the AA-metabolizing activity and nuclear localization as 15-LOX2sv-b, a splice variant that does not metabolize AA and is mostly excluded from nucleus, demonstrates similar inhibitory effect on PCa development.

MATERIALS AND METHODS

Cells and Reagents—Six primary NHP cell strains, NHP1–NHP6, were prepared from six different donors. NHP1, NHP3, NHP4, and NHP6 cells were obtained from Clonetics (Walkersville, MD), and NHP2 and NHP5 cells were generated as previously described (7–9). These cells were cultured in serum-free, PrEBM medium (Clonetics)

[1] The abbreviations used are: 15-LOX2, 15-lipoxygenase 2; 15-LOX2sv-a/b/c, 15-lipoxygenase 2 splice variant a, b, or c; AA, arachidonic acid; CAP, cytoskeleton-associated proteins; Cox-II, cytochrome oxidase subunit II; CSK, cytoskeleton; LDH, lactate dehydrogenase; NHP, normal human prostate epithelial cells; PCa, prostate cancer; NLS, nuclear localization signal; PPAR-γ, peroxisome proliferator-activated receptor-γ; WCL, whole cell lysate; DAPI, 4′,6-diamidino-2-phenylindole; 15(*S*)-HETE, 15(*S*)-hydroxyeicosatetraenoic acid; FBS, fetal bovine serum; GFP, green fluorescent protein; HM, heavy membrane; LM, light membrane; MES, 4-morpholineethanesulfonic acid; hrGFP, humanized *Renilla* GFP; IRES, internal ribosomal entry site; UT, untransfected; UG, urogenital; RT, reverse transcription; ER, endoplasmic reticulum; pCMV, cytomegalovirus promoter.

supplemented with insulin, epidermal growth factor, hydrocortisone, bovine pituitary extract, and cholera toxin, and used during passages 2–6 (3). PCa cell lines, *i.e.* PPC-1, PC3, and LNCaP, were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. HEK 293 cells were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium supplemented with 5% FBS and antibiotics.

Rabbit polyclonal anti-15-LOX2 antibody was described before (4). Rabbit polyclonal anti-E-cadherin and goat polyclonal anti-lamin A antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal anti-human vinculin (clone hVIN-1) was bought from Sigma (St. Louis, MO). Goat anti-lactate dehydrogenase (LDH) antibody was purchased from Chemicon (Chemicon International, Inc., Temecula, CA). Monoclonal anti-actin and anti-cytochrome *c* oxidase subunit II (Cox-II) antibodies were purchased from ICN (Indianapolis, IN) and BD Pharmingen (San Diego, CA), respectively. A monoclonal anti-BrdUrd (5-bromo-2′-deoxyuridine) antibody and a rabbit polyclonal anti-Bap31 antibody were kindly provided by Drs. M. Raff and G. Shore, respectively. Anti-GFP (green fluorescent protein) antibodies were obtained from Clontech (Palo Alto, CA). All secondary antibodies (goat anti-mouse or -rabbit IgG or rabbit anti-goat IgG conjugated to horseradish peroxidase, fluorescein isothiocyanate, or Rhodamine) were acquired from Amersham Biosciences (Piscataway, NJ). Liposome FuGENE 6 was bought from Roche Applied Science (Indianapolis, IN). All other chemicals were bought from Sigma unless specified otherwise.

*Immunohistochemistry of 15-LOX2 Expression in Tissue Sections—*Paraffin-embedded sections of normal prostate tissues and PCa were blocked for endogenous peroxidase activity with 3% H_2O_2 in water for 10 min. Antigen retrieval was done by incubating the slides with 10 mM citrate buffer (pH 6.0) for 10 min in a microwave oven. Slides were then blocked for nonspecific binding in 10% goat whole serum (30 min) followed by incubation in anti-15-LOX2 antibody (30 min, room temperature). Slides were finally incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase followed by substrate (dimethyl amino azobenzene) incubation.

*Immunofluorescence Detection of 15-LOX2 Expression in Cultured NHP Cells—*The basic procedure was as described previously (3). For double labeling of 15-LOX2 and E-cadherin or 15-LOX2 and vinculin, cells were first labeled for 15-LOX2 followed by goat anti-rabbit IgG conjugated to fluorescein isothiocyanate. After post-blocking in 15% goat whole serum, cells were incubated with antibodies against E-cadherin or vinculin followed by secondary antibody conjugated to Rhodamine.

*Western Blotting and Subcellular Fractionation—*Whole cell lysate (WCL) was prepared in TNC buffer (10 mM Tris acetate, pH 8.0, 0.5% Nonidet P-40, and 5 mM $CaCl_2$) or complete radioimmune precipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.5% Triton X-100, 10 mM EDTA) containing protease inhibitor mixture. The WCL prepared in TNC generally contains much lower nuclear, cytoskeletal, or cytoskeleton (CSK)-associated organelles (such as mitochondria) or proteins (3). Protein concentrations were determined by MicroBCA kit (Pierce, Rockford, IL). Samples containing same amounts of proteins were loaded on 15% SDS-PAGE and Western blotting performed using enhanced chemiluminescence (ECL).

Subcellular fractionation was carried out in log-phase NHP6 cells as previously described (10–13) with slight modifications. Briefly, heavy membrane (HM) and light membrane (LM) fractions and cytosol were prepared using homogenization combined with differential centrifugation. Nuclei were prepared using the NUCLEI EZ PREP kit (Sigma). To prepare CSK and CSK-associated proteins (CAP) (10), NHP6 cells were first lysed in TNC buffer by scraping. The Nonidet P-40-insoluble pellet was extracted (10 min, 3×) on ice with high salt, Triton-containing CSK extraction buffer (600 mM KCl, 1.0 mM $MgCl_2$, 50 mM MES, pH 7.6, 10 µg/ml DNase, 10 µg/ml RNase, 1% Triton X-100, and protease mixture). The Triton-resistant residue was designated as CSK, and the Triton-soluble portions from each extraction were pooled and proteins precipitated with an equal volume of ice-cold acetone (10). The resultant protein pellet was designated CAP (10). 50–100 µg of each subcellular fraction was used in Western blotting for 15-LOX2. Then the same membrane was stripped and reprobed for various marker proteins as detailed in the text.

*Establishing Stable PCa Cell Lines Expressing 15-LOX2 or 15-LOX2sv-b—*15-LOX2 or 15-LOX2 splice variant cDNAs (3) were subcloned into pIRES-hrGFP (Stratagene, La Jolla, CA), in which the target gene (*i.e.* 15-LOX2 or 15-LOXsv-a/b/c) is driven by pCMV and hrGFP (humanized *Renilla* green fluorescent protein) is transcribed from an internal ribosomal entry site (IRES). The resultant vectors

were designated p15-LOX2-hrGFP, p15-LOX2sv-a-hrGFP, p15-LOX2sv-b-hrGFP, and p15-LOX2sv-c-hrGFP, respectively. These vectors, along with pIRES-hrGFP empty vector, were first transiently transfected into 293 cells to characterize their expressions. To establish stable clones, PC3 or LNCaP cells were co-transfected with pIRES-hrGFP, p15-LOX2-hrGFP, or p15-LOX2sv-b-hrGFP and pCMV-neo (Invitrogen) as a selectable marker. 48 h after transfection, G418 was added to the medium (800 µg/ml for LNCaP and 1 mg/ml for PC3 cells, respectively). Two weeks later, antibiotic-resistant PC3 cells were harvested and plated at clonal density (*i.e.* 50–100 cells/10-cm dish) and individual GFP-positive clones were selected, under an inverted fluorescence microscope, using a cloning ring. For LNCaP cells, stable clones were established by first enriching GFP-positive cells using fluorescence-activate cell sorting, followed by a limiting dilution method in 96-well culture plates. Two to four stable clones of each cell type were propagated and characterized by both Western blotting and immunofluorescence microscopy.

*Determination of 15-HETE Production in Stably Transfected PCa Cells by Liquid Chromatography and Tandem Mass Spectrometry—*Untransfected LNCaP or PC3 cells, or these cells stably transfected with pIRES-hrGFP, p15-LOX2-hrGFP, or p15-LOX2sv-b-hrGFP, were used to measure 15(*S*)-HETE production as previously detailed (3).

*Effect of 15-LOX2 Expression on PCa Cell Proliferation—*Untransfected PC3 cells or stable PC3 cell transfectants (passage 8) were plated, in quadruplicate, in 24-well flat-bottom culture plates at 5000 cells/well. The cells were cultured in RPMI medium containing 1, 2, or 5% FBS. In some conditions, AA at 1–25 µM was added in the culture medium. 72 h after plating, the numbers of dead and live cells in each well were determined by harvesting both floating and adherent cells and counting using the trypan dye exclusion assays (9). The results were expressed as a percentage of the control, and the experiment was repeated three times.

*Effect of 15-LOX2 Expression on PCa Development in Vivo—*Surgical orthotopic implantation was carried out to assess the effect of restoration of 15-LOX2 expression on PCa development *in vivo*. The basic procedure was previously described (14). Briefly, animals were anesthetized by intraperitoneal injection of Nembutal Mix (10 µl/g of body weight). Four groups of PC3 cells, *i.e.* untransfected (UT) or cells transfected with pIRES-hrGFP (GFP), p15-LOX2-hrGFP (15-LOX), or p15-LOX2sv-b-hrGFP (15-LOX2sv-b), all at passage 8, were orthotopically injected into athymic NCr-nu (The Jackson Laboratory, Bar Harbor, ME) nude mouse prostate (2×10^6 in 25 µl of RPMI/prostate). Tumor development was monitored 2 weeks after surgical implantation. About 2 months (*i.e.* 63 days) after implantation, the experiment was terminated, animals were sacrificed, and primary tumors together with the urogenital (UG) organs except bladder were dissected out. Tumor weights (with UG organs) were determined, and prostates from all four groups were used in H-E staining and immunohistochemical analysis.

*Nuclear Localization of 15-LOX2 and Its Splice Variants—*PC3 or LNCaP cells grown on glass coverslips were either untransfected or transiently transfected with various vectors using FuGENE 6 (3). Cells were fixed 48 h after transfection and then processed for 15-LOX2 staining (3). The distribution of 15-LOX2 in the transfected (*i.e.* GFP$^+$) cells was observed under a fluorescence microscope. In some cases, stable transfectants of PC3 and LNCaP cells were used in similar studies. In other experiments, cells were used in subcellular fractionation.

*Site-specific Mutagenesis of 15-LOX2 and Nuclear Localization Studies—*Site-specific mutagenesis was performed to change the 15-LOX2 R203K204, K214R215, and R220R221 to A203S204, R214S215, and A220S221, respectively, using the QuikChange site-specific mutagenesis system (Stratagene) and p15-LOX2-hrGFP as template. A triple mutant was also made. The successfully mutated sequences were confirmed by restriction digestion and sequencing analysis. These 15-LOX2 mutants, along with 15-LOX2 and 15-LOX2sv-a/b expression constructs, were transiently transfected into PC3 cells, and, 48 h later, cells were processed for 15-LOX2 staining.

*RT-PCR Analysis of the mRNA Levels 15-LOX2 and Its Splice Variants and Mutants—*Log-phase LNCaP cells were transfected with pIRES-hrGFP, p15-LOX2-hrGFP, p15-LOX2sv-a-hrGFP, p15-LOX2sv-b-hrGFP, p15-LOX2sv-c-hrGFP, or four NLS mutants mentioned above. 48 h after transfection, cells were selected by adding G418 (800 µg/ml). Ten days later, these G418-selected LNCaP cells, together with untransfected PC3 cells or PC3 stable clones, were harvested for RT-PCR analysis. Total RNA was isolated with the RNeasy Mini kit (Qiagen), and 0.6 µg of the total RNA was used in RT-PCR analysis using the MasterAmp One-Step RT-PCR kit (Epicenter, Madison, WI). Primers C (5′-ACTACCTCCCAAAGAACTTCCCC-3′, forward) and D (5′-
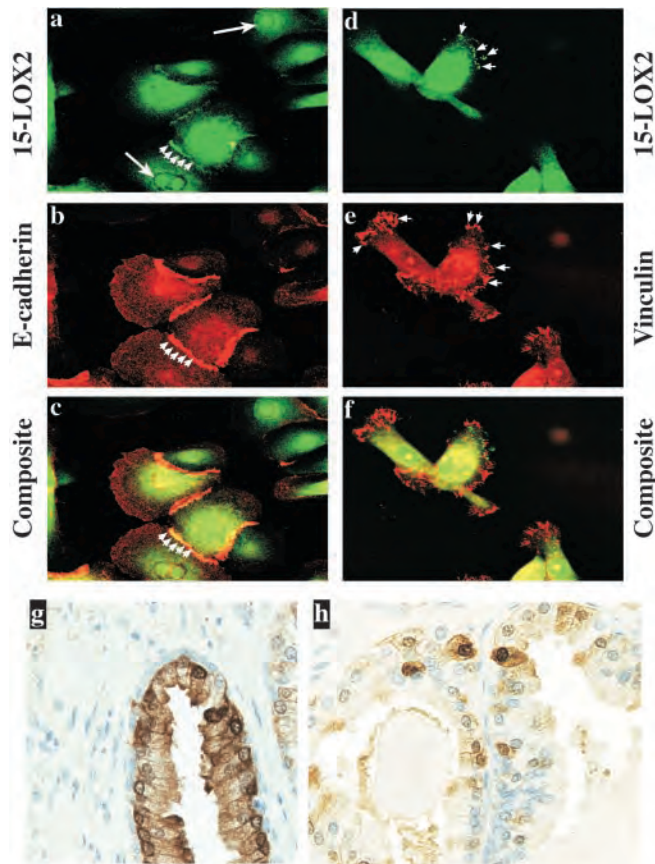
FIG. 1. **Immunofluorescent and immunohistochemical analysis of 15-LOX2 expression in NHP cells *in vitro* and benign prostate epithelial cells *in vivo*.** *a–f*, NHP2 (P5) grown on glass coverslips were double-labeled with 15-LOX2 and E-cadherin (*a* and *b*, respectively) or with 15-LOX2 and vinculin (*d* and *e*, respectively). *c* and *f* are composite images. *Small arrows* indicate the cell-cell border localization of 15-LOX2, whereas the *large arrows* are the nuclear localization. Note the colocalization of 15-LOX2 with E-cadherin (*a–c*) but not with vinculin (*d–f*). *g* and *h*, prostate tissue sections of normal (*g*) or PIN (*h*) glands stained for 15-LOX2 (*brown*). Note clear staining at the cell-cell border as well as in the nuclei in addition to cytoplasmic staining in both images. Original magnifications, ×400 for *a–f* and ×100 for *g* and *h*.

TTCAATGCCGATGCCTGTG-3′, reverse) were used to amplify 15-LOX2 as previously described (3). This pair of primers amplifies 15-LOX2 and 15-LOX2sv-c as a 546-bp band and 15-LOX2sv-a and 15-LOX2sv-b as a 459-bp band (3). RT-PCR of glyceraldehyde-3-phosphate dehydrogenase was used as a control (3). Plasmids (1 ng) were used as positive controls.

Statistical Analysis—Student's *t* test was used to determine the statistical differences between various experimental groups with $p < 0.05$ considered significantly different.

RESULTS

15-LOX2 Is Expressed in the Nucleus and Other Subcellular Locations—15-LOX2 is a negative cell-cycle regulator in NHP cells (3). In an attempt to understand its molecular mechanisms of action, we studied its subcellular expression in cultured primary NHP cells as well as in benign prostate epithelial cells *in vivo*. As observed previously (3), 15-LOX2 was primarily expressed in the cytoplasm. However, significant amounts of 15-LOX2 were also localized at the cell-cell borders (Fig. 1*a*, *small arrows*) as well as in the nuclei (Fig. 1*a*, *large arrows*). The 15-LOX2 distributed at the cell-cell borders partially co-localized with the adhesion molecule E-cadherin (Fig. 1, *a–c*). In some cells, 15-LOX2 was also observed as discrete dots or clusters at the cell periphery (Fig. 1*d*, *arrows*) resembling cell-matrix interaction sites called focal adhesions (15).
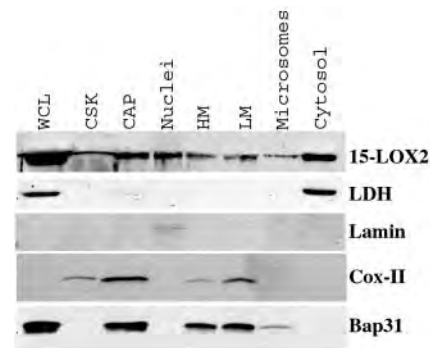

FIG. 2. **Analysis of 15-LOX2 expression by subcellular fractionation.** NHP 6 (P5) cells were fractionated into CSK, CAP, nuclei, HM, LM, microsomes, and cytosol, as detailed under "Materials and Methods." WCL (prepared in TNC buffer) was used as control. Proteins from each fraction (50 μg for CSK and microsomes and 100 μg for all other fractions) were separated on 15% SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for 15-LOX2 and then for various marker proteins as indicated (see text). *Cox-II*, cytochrome *c* oxidase subunit II; *LDH*, lactate dehydrogenase.

Double staining of 15-LOX2 and vinculin, a protein marker for focal adhesions (15), however, did not reveal any co-localization (Fig. 1, *d–f*). *In vivo*, 15-LOX2 was also expressed in the cytoplasm, cell-cell borders, as well as in the nuclei (Fig. 1*g*). Note that, as previously reported (4), 15-LOX2 was specifically expressed in the glandular prostate epithelial cells *in vivo* but not in basal cells or other cell types including stromal cells (Fig. 1*g*). Also, as noted previously (5), 15-LOX2 staining was reduced in the precursor lesion PIN (prostate intraepithelial neoplasia), and most cells in these lesions homogeneously lost the 15-LOX2 staining (Fig. 1*h*). However, prominent cell membrane and cell-cell border staining, and, in particular, nuclear staining was still evident in some 15-LOX2-positive cells (Fig. 1*h*).

To confirm the subcellular distribution pattern of 15-LOX2 biochemically, we carried out a fractionation analysis (10–13). NHP6 cells were fractionated into CSK, CAP, nuclei, HM (the $1000 \times g$ pellet containing mainly large mitochondria, plasma membrane sheets, and small amounts of other organelles (13, 16)), LM (the $10,000 \times g$ pellet containing mainly smaller mitochondria and some lysosomes and peroxisomes (13, 16)), microsomes (*i.e.* the $100,000 \times g$ pellet containing ER, Golgi, endosomes, and membrane skeleton (12, 16)), and cytosol (*i.e.* the $100,000 \times g$ supernatant (13)). WCL was used as a control. As shown in Fig. 2, consistent with the immunostaining data (Fig. 1), 15-LOX2 was primarily detected in the cytosol, but significant amounts of 15-LOX2 were also detected in the nuclei and CAP. Lower yet easily detectable levels of 15-LOX2 were also observed in all other fractions, including CSK, HM, LM, and microsomes (Fig. 2). As expected, the highest amount of 15-LOX2 was detected in WCL. The purity of each fraction was confirmed by specific markers. For instance, lactate dehydrogenase (LDH), a cytosolic marker (16), was detected only in the cytosol (Fig. 2), suggesting that there was no contamination of all other subcellular fractions by the cytosol. Similarly, lamin A, a nuclear intermediate filament, was detected only in the nuclei. Cytochrome *c* oxidase subunit II (Cox-II), a mitochondrial inner membrane respiratory complex protein, was detected, as expected, most prominently in CAP and also in CSK (Fig. 2), because most mitochondria normally are associated with microtubules and some other cytoskeletal elements (17). Cox-II was also detected, expectedly, in the HM and LM fractions (Fig. 2), which normally are enriched with the mitochondria (11, 13). Note that no lamin A or Cox-II was detected in WCL, probably due to the low levels of nuclei and mitochondria
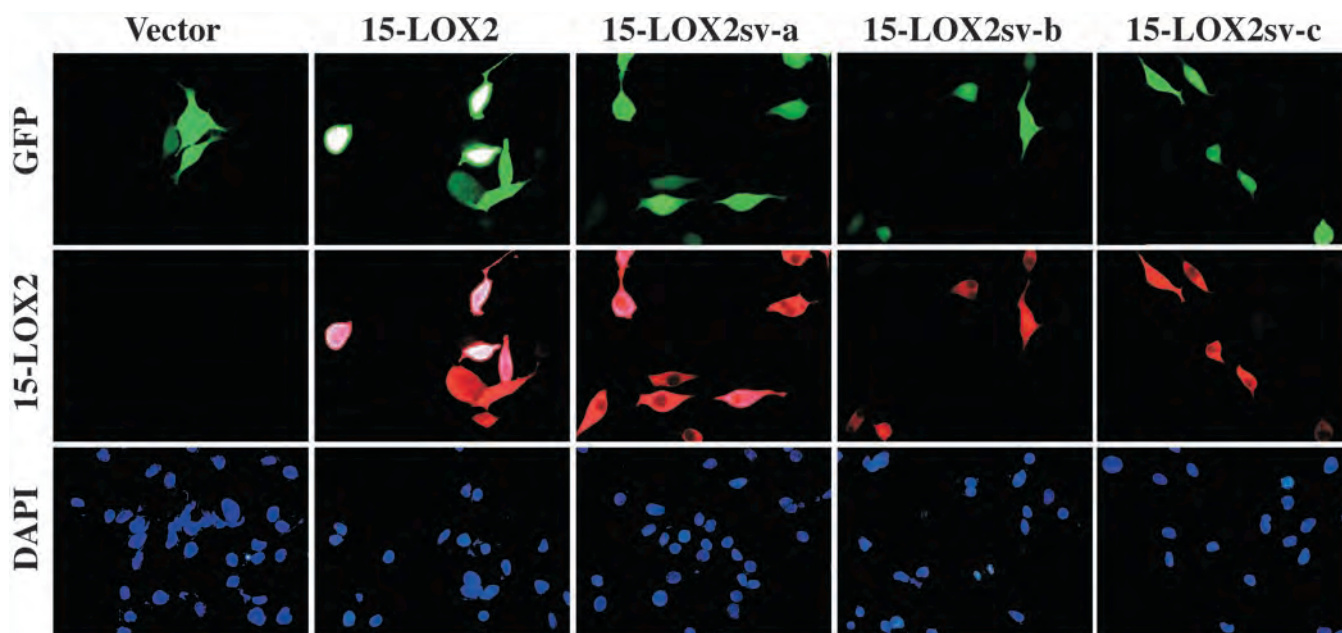
FIG. 3. **Lack of nuclear localization of 15-LOX2 splice variants.** LNCaP cells were transiently transfected with the vector (*pIRES-hrGFP*), p15-LOX2-IRES-hrGFP (*15-LOX2*), p15-LOX2sv-a-IRES-hrGFP (*15-LOX2sv-a*), p15-LOX2sv-b-IRES-hrGFP (*15-LOX2sv-b*), or p15-LOX2sv-c-IRES-hrGFP (*15-LOX2sv-c*). 48 h post transfection, cells were processed for 15-LOX2 immunostaining and nuclei were counterstained by 4′,6-diamidino-2-phenylindole (*DAPI*). Shown are the representative microphotographs of GFP, 15-LOX2, and DAPI images from three independent experiments with comparable results. Over several thousands of cells analyzed, 15-LOX2 splice variants were clearly excluded from the nucleus in the majority (>95%) of the cells, although the strong transgene expression in some cells tended to mask their non-nuclear expression pattern. Original magnifications, ×200.

in the WCL prepared using the TNC buffer (see "Materials and Methods"). Finally, Bap31, an integral ER membrane protein (18), was detected in CAP, HM, LM, microsomes, and WCL, but not in the cytosol, nuclei, or CSK (Fig. 2).

Collectively, data in Figs. 1 and 2 indicate that, in addition to its predominant expression in the cytosol, 15-LOX2 is also expressed at multiple other subcellular locations, including nuclei, cell-cell borders, CSK, and membrane fractions.

None of the Three 15-LOX2 Splice Variants Is Localized to the Nucleus—The nuclear localization of 15-LOX2 is particularly interesting, because it suggests that the molecule may play a distinct signaling function in the nucleus. Therefore, our subsequent studies focused on the nuclear localization of 15-LOX2 and its relationship with the enzymatic and functional activities. We previously cloned three 15-LOX2 splice variants termed 15-LOX2sv-a/b/c (3). These splice variants have spliced out some critical amino acid residues important for the AA-metabolizing enzymatic activities (2, 3). To determine whether these splice variants are also localized in the nucleus, we transiently transfected various expression plasmids into LNCaP cells, which do not express readily detectable levels of 15-LOX2. As shown in Fig. 3, although 15-LOX2 was distributed throughout the cells, including the nucleus as confirmed by subcellular fractionation (not shown), all three splice variants were mostly excluded from the nucleus. Identical results were observed in stably transfected LNCaP (Fig. 4, *a–d*) or PC3 cells (Fig. 4, *e–h*). It should be pointed out that the obvious lack of nuclear staining of 15-LOX2 splice variants was not due to overall reduced protein expression, because comparable levels of 15-LOX2 and its splice variants were observed in multiple experiments of either transiently (*e.g.* Fig. 3) or stably (*e.g.* Fig. 4) transfected PCa cells. A typical example is shown in Fig. 4, in which LNCaP cells stably transfected with 15-LOX2 or 15-LOX2sv-b (Fig. 4, *b* and *d*) or PC3 cells stably transfected with 15-LOX2 or 15-LOX2sv-b (Fig. 4, *f* and *h*) showed very similar levels of protein expression (also see Figs. 5 and 7*b* and the discussion below).

A Putative Nuclear Localization Signal in 15-LOX2 Is Insufficient for Its Nuclear Targeting—Transport between the nucleus and the cytoplasm occurs through the nuclear pore complex on the nuclear envelope, and proteins can enter the nucleus either by diffusion or by signal-mediated transport (19). Generally, only proteins with masses <40 kDa are able to enter the nucleus by passive diffusion (19). Signal-mediated nuclear transport requires energy, optimal temperature, a NLS, and soluble transport machinery (19). Two of the best characterized NLSs are the SV40 large T NLS (often called the classic monopartite NLS), which is composed of a stretch of basic amino acids, and the nucleoplasmin bipartite NLS, which is composed of two basic stretches or clusters separated by 9–12 amino acid residues (19, 20). Recent studies have also revealed other potential NLS (*e.g.* glycine-rich sequences) that do not conform to these two motifs (19, 20).

Because a significant portion of 15-LOX2 is localized in the nucleus, we reason that there may exist one or more specific NLSs in the molecule responsible for its nuclear targeting. Therefore, we looked for a potential NLS in 15-LOX2 by searching an available data base (cubic.bioc.columbia.edu/predictNLS (20)) and by using tools such as PROSITE and MotifScan. We did not find any credible stretch of basic amino acids that would correspond to the monopartite NLS. However, we did uncover a potential bipartite NLS, [203]RKGLWRSLNEMKRIFNFRR[221], which is located at the N terminus of 15-LOX2. To determine whether this putative NLS plays a role in the nuclear import of 15-LOX2, we used site-specific mutagenesis to mutate the three di-basic amino acid sequences. As shown in Fig. 5, 15-LOX2 transfected into PC3 cells was localized throughout the cells, including nuclear area (*a–c*), whereas both 15-LOX2sv-a and 15-LOX2sv-b were mostly excluded from nuclei (*d–i*). Compared with 15-LOX2-transfected PC3 cells, cells transfected with the 15-LOX2 mutants, *i.e.* 15-LOX2RK/AS (Fig. 5, *j–l*), 15-LOX2KR/RS (Fig. 5, *m–o*), 15-LOX2RR/AS (Fig. 5, *p–r*), or triple mutant (not shown), showed partially reduced nuclear staining. Most cells transfected with the 15-LOX2 mutants
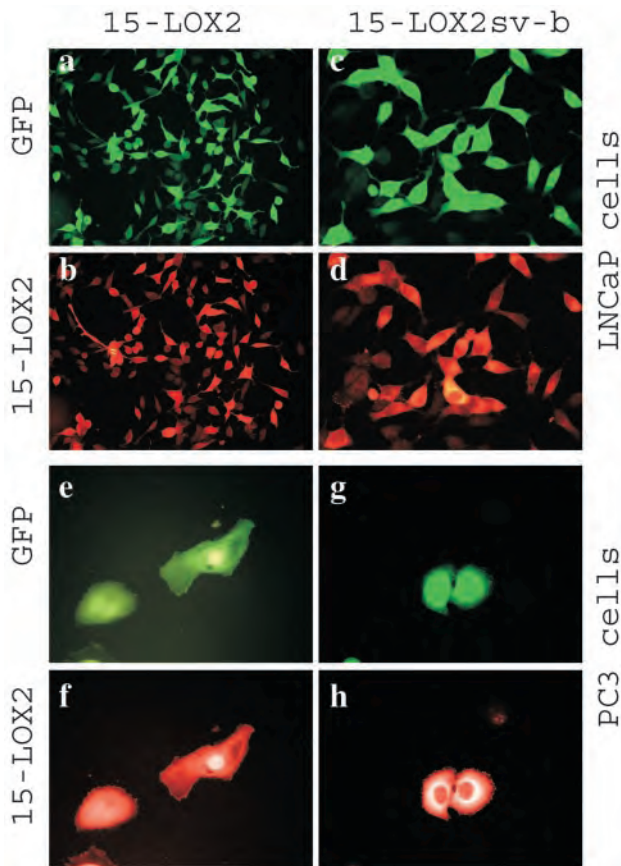
FIG. 4. **Nuclear exclusion of 15-LOXsv-b and similar protein levels of 15-LOX2 and 15-LOX2sv-b in stably transfected PCa cells.** *a–d*, a clone (clone 1) of LNCaP cells stably transfected with p15-LOX2-hrGFP (*a* and *b*) or p15-LOX2sv-b-hrGFP (*c* and *d*) was plated on glass coverslips and processed for immunofluorescent staining using the rabbit polyclonal anti-15-LOX2 antibody (3). Shown are the representative images of GFP (*a* and *c*) and 15-LOX2 (*b*) or 15-LOX2sv-b (*d*). Note that images in *c* and *d* were enlarged to show the non-nuclear expression pattern of 15-LOX2sv-b. Original magnifications: *a* and *b*, ×200; *c* and *d*, ×400. *e–h*, a clone (clone 1) of PC3 cells stably transfected with p15-LOX2-hrGFP (*e* and *f*) or p15-LOX2sv-b-hrGFP (*g* and *h*) was processed for immunofluorescent staining. Shown are the representative images of GFP (*e* and *g*) and 15-LOX2 (*f*) or 15-LOX2sv-b (*h*). Original magnifications: ×400. Untransfected LNCaP or PC3 cells, or LNCaP or PC3 cells stably transfected with pIRES-hrGFP, showed no 15-LOX2 staining (not shown; also see Fig. 3 and Ref. 3). Note that 15-LOXsv-b is mostly excluded from the nucleus in both LNCaP (*d*) and PC3 (*h*) cells and that similar protein levels of 15-LOX2 and 15-LOX2sv-b were observed in stably transfected PCa cells (compare *d versus b* or *h versus f*).

FIG. 5. **Partial involvement of the putative NLS (²⁰³RKGL-WRSLNEMKRIFNFRR²²¹) in the import of 15-LOX2 to the nucleus.** The *underlined* RK, KR, and RR sequences were mutated individually or in combination as described under "Materials and Methods." The respective expression plasmids, along with 15-LOX2, 15-LOX2sv-a, or 15-LOX2sv-b vectors, were transfected into PC3 cells. Shown are the representative microphotographs of 15-LOX2 (*a, d, g, j, m,* and *p*), GFP (*b, e, h, k, n,* and *q*), and DAPI (*c, f, i, l, o,* and *r*) images. Note that 15-LOX2sv-a (*d*) and 15-LOX2sv-b (*g*) were excluded from the nucleus in most cells, whereas 15-LOX2 was expressed throughout the cell, including the nuclear area (*a*). The RK/AS (*j*), KR/RS (*m*), and RR/AS (*p*) mutants and the triple mutant (not shown) showed reduced nuclear staining (*arrows*). Asterisks in *a, d, g, j,* and *m* illustrate several transfected 15-LOX2-positive cells that are only weakly positive or negative for GFP, probably because GFP was translated downstream of 15-LOX2 through IRES. The images are representative of the results from two independent experiments. Original magnifications: ×200.

showed a nuclear staining intensity between those of 15-LOX2 and 15-LOX2sv-a/b (*e.g.* Fig. 5, *j, m,* and *p*; *arrows*). These observations suggest that the Arg²⁰³–Arg²²¹ NLS is only partially involved in the nuclear import of 15-LOX2.

Similar to the 15-LOX2 splice variants transfected into PCa cells (Figs. 3 and 4), the 15-LOX2 NLS mutants transfected into PC3 cells also showed levels of protein expression comparable to that of 15-LOX2 on immunofluorescence staining (Fig. 5). Because the transient transfection efficiency varied greatly with different expression constructs and the efficiency (1–10%) generally did not allow us to quantify the protein levels by Western blotting, we adopted a different approach to analyze the mRNA levels of 15-LOX2 and its variants or NLS mutants transfected into PCa cells. For this purpose, LNCaP cells were first transiently transfected with various expression constructs followed by selection with G418 for 10 days. At the end of the selection, the majority of G418-resistant cells were GFP-positive, and these enriched cells were then used in RT-PCR anal-
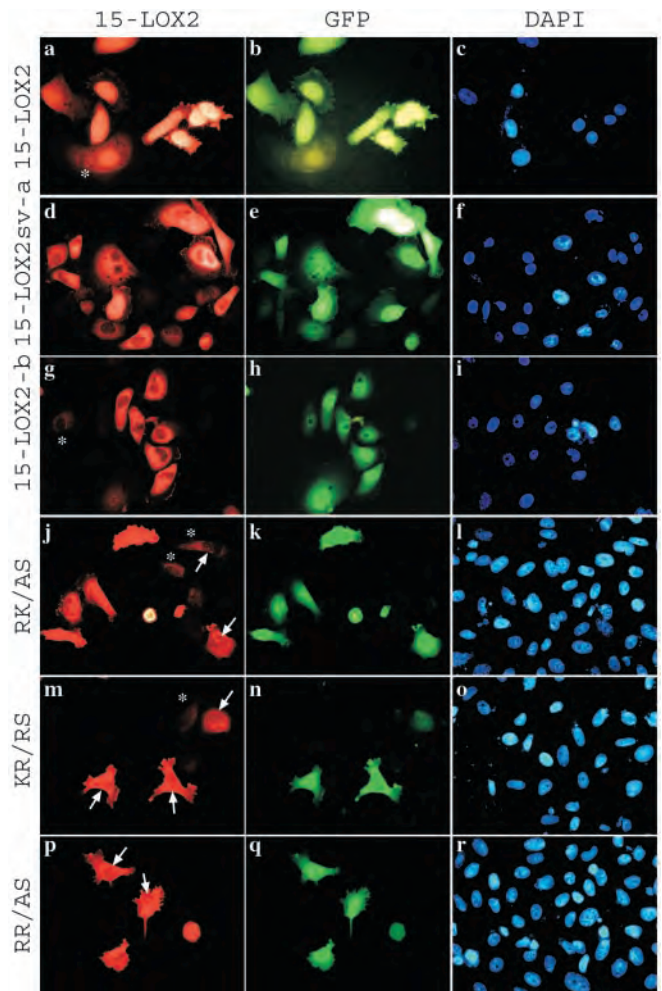
ysis using a pair of primers that could pick up 15-LOX2 and all its three splice variants (3). As shown in Fig. 6, untransfected LNCaP cells and LNCaP cells transfected with pIRES-hrGFP did not express 15-LOX2 or any splice variant, consistent with previous observations (3) as well as with protein data (*e.g.* Fig. 3). In contrast, LNCaP cells transfected with 15-LOX2 or its splice variants or NLS mutants showed overall similar mRNA levels (Fig. 6; data not shown for 15-LOX2sv-c and the NLS triple mutant). In fact, we consistently observed slightly higher mRNA levels for most 15-LOX2 splice variants or mutants (Fig. 6). These results are consistent with our immunofluorescence data that show similar protein levels of 15-LOX2 and its variants or mutants transfected into the PCa cells.

Restoration of 15-LOX2 Expression Inhibits PCa Cell Proliferation in Vitro and Prostate Tumor Development in Vivo: 15-LOX2sv-b Also Demonstrates Significant Inhibitory Effect—
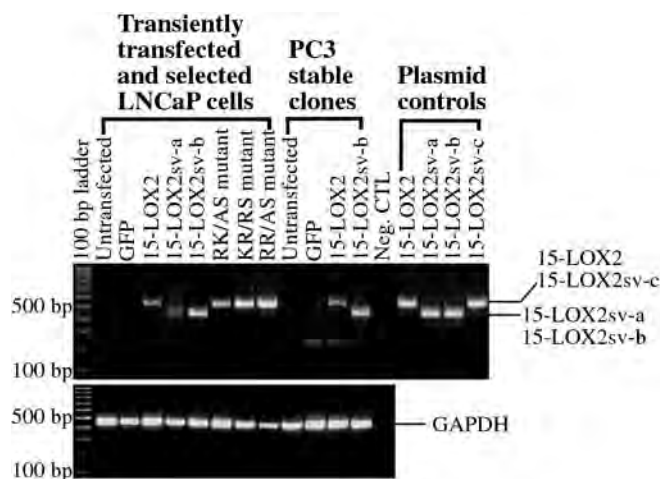
FIG. 6. **Similar levels of mRNA expression of 15-LOX2 and its splice variants or NLS mutants transfected into PCa cells.** LN-CaP cells were transiently transfected with various expression constructs, selected for using G418, and then used for RNA extraction and RT-PCR analysis, as detailed under "Materials and Methods." PC3 stable clones were also analyzed for mRNA expression. The RT-PCR was performed using C-D primers, which amplify 15-LOX2 and 15-LOX2sv-c as a 546-bp band and 15-LOX2sv-a and 15-LOX2sv-b as a 459-bp band (3). RT-PCR of glyceraldehyde-3-phosphate dehydrogenase was used as a control (3). The respective plasmids (the *last four lanes*; 1 ng each) were used as positive controls.

Most PCa cells demonstrate reduced or lost expression of 15-LOX2 (3–6), suggesting that 15-LOX2 may represent an endogenous prostate tumor suppressor. To directly test this hypothesis, we started by attempting to establish PCa cell lines (PPC-1 and LNCaP) stably expressing 15-LOX2 using the pCMS expression constructs (3), in which 15-LOX2 or its splice variants are driven by the CMV promoter, whereas the EGFP module is driven by the SV40 promoter. Multiple experiments indicated that, although we could initially establish stable clones expressing both 15-LOX2 (or splice variants) and GFP, expression of 15-LOX2 or its splice variants was preferentially lost starting from passage 3 (not shown). These results are consistent with the concept that 15-LOX2, and perhaps its splice variants as well, are inhibitory to PCa cells.

We then made expression constructs in the pIRES-hrGFP vector, in which the transcription of both 15-LOX2 (or splice variants) and hrGFP is controlled by the same CMV promoter and translation of hrGFP is initiated from an internal ribosomal entry site (IRES). When transiently transfected into 293 (not shown) or PCa cells (Figs. 3–6), the expected protein products were detected by immunofluorescence and/or Western blotting. We then used these constructs and established stable PC3 and LNCaP clones expressing 15-LOX2 or 15-LOX2sv-b. Of the several hundred GFP[+] clones transfected with 15-LOX2 or 15-LOX2sv-b that we screened, only ~1% of the cells could be made into long term stable clones. By contrast, ~60% of GFP^{+} cells transfected with hrGFP alone could become stable clones. These observations are also consistent with the 15-LOX2 being inhibitory to PCa cells.

Shown in Fig. 7a is one clone of PC3 cells expressing 15-LOX2, 15-LOX2sv-b, or GFP alone. Nearly all cells in the clone were GFP-positive but only the cells stably transfected with 15-LOX2 or 15-LOX2sv-b were double positive for 15-LOX2 and GFP (Fig. 7b). Again, 15-LOX2 was expressed in the whole cell, including the nucleus, but 15-LOX2sv-b was mostly excluded from the nucleus as revealed by both immunolabeling (Fig. 7b) and subcellular fractionation (Fig. 7c). Similar results were observed with several other PC3 cells clones as well as with stable LNCaP clones (not shown). Note that in both Western
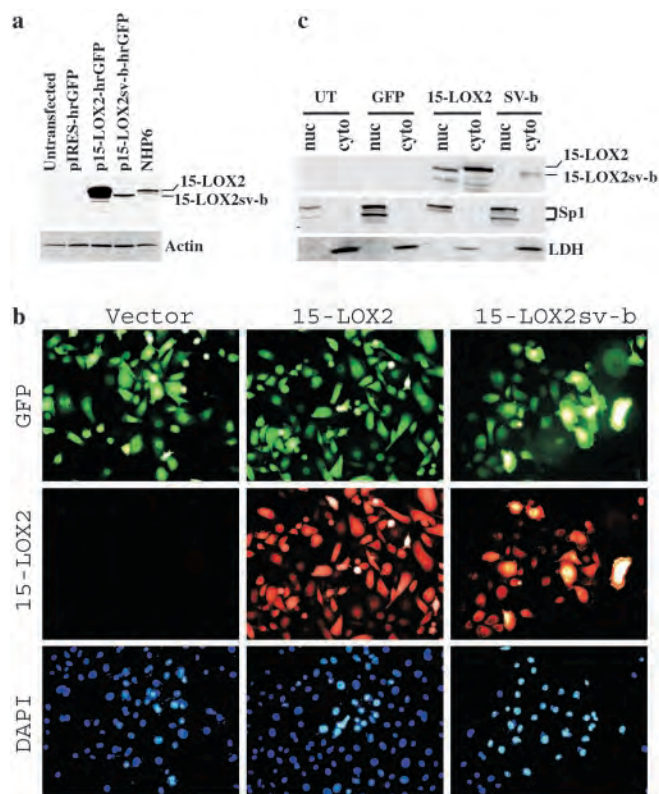

FIG. 7. **Establishment of stable PC3 cell clones expressing 15-LOX2 or 15-LOX2sv-b.** a, Western blotting of 15-LOX2 and 15-LOX2sv-b in a stable clone of PC3 cells (passage 6) using 30 μg of whole cell lysate. The 15-LOX2 and 15-LOX2sv-b protein bands were indicated on the *right*. NHP6 (passage 5) cells were used as a positive control. b, the same clone of PC3 cells (as shown in a) stably transfected with pIRES-hrGFP (*Vector*), p15-LOX2-IRES-hrGFP (*15-LOX2*), or p15-LOX2svb-IRES-hrGFP (*15-LOX2sv-b*), respectively, were stained for 15-LOX2 and nuclei (*DAPI*). Original magnifications, ×200. c, nuclear localization of 15-LOX2 but not 15-LOX2sv-b in stably transfected PC3 cells. Subcellular fractionation was carried out as described under "Materials and Methods," and 60 μg of nuclear (*nuc*) or cytosolic (*cyto*) proteins/lane was separated on a 15% SDS-PAGE. After transfer, the membrane was probed for 15-LOX2, stripped, and then reprobed for Sp1 proteins (as a nuclear marker; the *upper bands* being the phosphorylated Sp1) or LDH. Note that several *lower bands* were consistently detected in both cytosolic and nuclear fractions from the cells transfected with 15-LOX2, which might be degradation products.

blotting (Fig. 7a) and subcellular fractionation (Fig. 7c), we observed lower protein levels of 15-LOX2sv-b than 15-LOX2. Similar differences were also observed in transiently transfected 293 cells (3) as well as in other stable clones of PC3 and LNCaP cells (not shown). This difference was unlikely due to differential protein expression as we consistently observed, on immunofluorescence microscopy, very similar protein levels of 15-LOX2 and its splice variants or NLS mutants (Figs. 3–5 and 7b). More importantly, we observed similar levels of 15-LOX2 and 15-LOX2sv-b mRNA in the stably transfected PC3 cells (Fig. 6). These observations, together, suggest that the polyclonal anti-15-LOX2 antibody preferentially recognizes 15-LOX2 and does not recognize its splice variants well on Western blotting (*e.g.* Fig. 7, *a* and *c*), although it recognizes equally well the undenatured proteins of 15-LOX2 and its variants or NLS mutants in immunofluorescent staining (*e.g.* Figs. 3–5, and 7b). This conclusion is also supported by our multiple experiments with transiently transfected 293 cells as well as with other PCa stable clones (3; data not shown). We are currently developing 15-LOX2 isoform-specific antibodies to directly address this issue.

As expected, untransfected PC3 and LNCaP cells, as well as

TABLE I
15(S)-HETE production in stably transfected PCa cells

15(S)-HETE production was measured in lysates from log-phase cells, in the presence of exogenous AA (100 μM; 37 °C $\times$ 10 min) using LC/MS/MS analysis as previously described (3). Data were obtained from two separate experiments and the values are mean $\pm$ S.D derived from two to three samples with each cell type.

Cells	15(S)-HETE level
	ng/10^6 cells
PC3	
Untransfected	0.63 $\pm$ 0.18
GFP	1.33 $\pm$ 0.15
15-LOX2	27.95 $\pm$ 3.16[a]
15-LOX2sv-b	1.77 $\pm$ 0.23
LNCaP	
Untransfected	0.85 $\pm$ 0.02
GFP	0.73 $\pm$ 0.05
15-LOX2	13.42 $\pm$ 0.25[a]
15-LOX2[b]	0.024 $\pm$ 0.002
15-LOX2sv-b	0.84 $\pm$ 0.06

[a] $p < 0.001$ (Student t test).
[b] 15(S)-HETE measurement in the absence of exogenous AA.

PC3 and LNCaP cells, transfected with GFP vector alone produced little 15(S)-HETE (Table I), because they do not express appreciable 15-LOX2 (3). By contrast, cells transfected with 15-LOX2 produced a significant amount of 15(S)-HETE (Table I). In contrast to 15-LOX2-transfected cells, cells transfected with 15-LOX2sv-b, in which two exons have been spliced out (3), produced little 15(S)-HETE (Table I). These measurements were done in the presence of added substrate, AA. In the absence of exogenous AA, the 15-LOX2-transfected LNCaP stable clones produced no 15(S)-HETE (Table I), suggesting that there was very little free AA in the cells under the normal culture conditions. Collectively, these data suggest that the 15-LOX2 in the stably transfected PCa cells is enzymatically active (*i.e.* capable of metabolizing AA), whereas the 15-LOX2sv-b is not.

To assess the effect of 15-LOX2 re-expression on PCa development, we first performed a cell proliferation assay using the stable clones. Consistent with our previous transient transfection experiments (3), PC3 cells stably expressing 15-LOX2 expression proliferated slower than either untransfected cells or the vector-transfected cells (Fig. 8a). Surprisingly, PC3 cells stably expressing 15-LOX2sv-b, which does not possess AA-metabolizing activity and is mostly excluded from the nucleus (see above), also showed slower cell proliferation (Fig. 8a). The inhibitory effect of 15-LOX2 and 15-LOX2sv-b was observed in either 1% or 5% FBS (Fig. 8a). As previously observed (3), re-expression of 15-LOX2 or 15-LOX2sv-b by itself did not affect apoptosis in the transfected cells, which were all healthy (*e.g.* Figs. 3–6, and 7b). However, in the presence of exogenous AA, the 15-LOX2 stable clones, but not 15-LOX2sv-b clones, showed a significant increase in apoptosis (not shown). For example, in the presence of 5 μM AA (72 h), only 14% of the PC3 cells stably transfected with 15-LOX2 were alive, compared with 88%, 70%, and 65% survivability in untransfected and PC3 cells stably transfected with GFP or 15-LOX2sv-b, respectively. These results, consistent with previous observations that high doses of 15(S)-HETE induce cell death in PCa cells (3, 21), suggest that the exogenously added AA is metabolized by transfected 15-LOX2 but not 15-LOX2sv-b to produce 15(S)-HETE, which in turn induces cell death.

Next, we carried out an orthotopic tumor implantation experiment in which PC3 cells stably expressing 15-LOX2 or 15-LOX2sv-b or the vector alone were injected into the mouse prostate. The experiment was terminated 63 days post tumor cell inoculation. As shown in Fig. 8 (b and c), the PC3 tumors
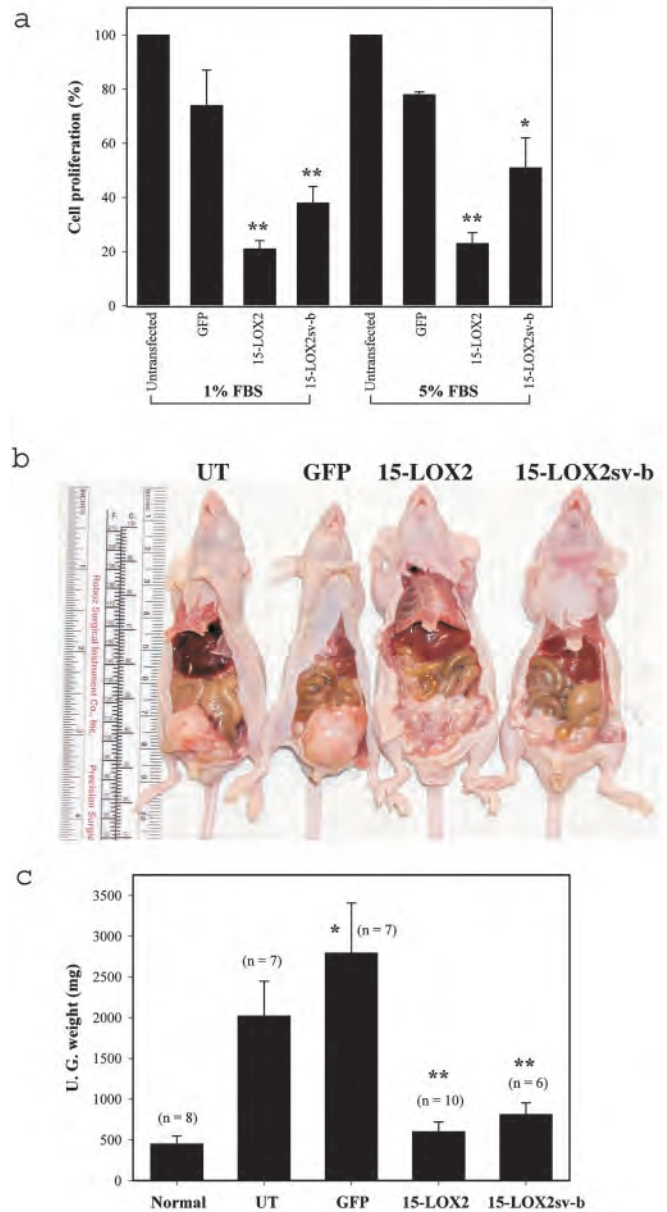
FIG. 8. **Inhibition of PC3 cell proliferation *in vitro* (a) and tumor development *in vivo* (b and c) by restoration of 15-LOX2 expression.** In a, proliferation of untransfected PC3 cells 72 h after plating was used as the baseline and considered 100%. Proliferation of PC3 cells stably transfected with pIRES-hrGFP (*GFP*), p15-LOX2-IRES-hrGFP (*15-LOX2*), or p15-LOX2sv-b-IRES-hrGFP (*15-LOX2sv-b*) was presented as percent proliferation of the untransfected PC3 cells. The *bars* represent the mean $\pm$ S.D. derived from three independent experiments. *, $p < 0.01$; **, $p < 0.001$. Note that the GFP-transfected stable PC3 cells also proliferated slightly slower (statistically insignificant) than the untransfected controls, as previously observed (3). In b, large solid tumors can be easily seen in the UT (untransfected) and GFP groups, whereas the 15-LOX2 and 15-LOX2sv-b groups showed minimal tumor burden. In c, the UG (urogenital) weights in the UT and GFP groups are significantly higher ($p < 0.001$) than the uninjected prostates (normal). For unknown reasons, tumors in the GFP group are larger than those in the UT group (*, $p < 0.05$; also see b). In contrast, tumors in both the 15-LOX2 and 15-LOX2sv-b groups are significantly (**, $p < 0.001$) smaller than tumors in either the UT or GFP group. The numbers (*n*) of animals in each group are indicated in the *parentheses*.

bearing 15-LOX2 were significantly smaller than the tumors bearing empty vector (*i.e.* GFP), suggesting that 15-LOX2 re-expression suppresses orthotopically implanted prostate tumor growth *in vivo*. Surprisingly and in support of the *in vitro* data (Fig. 8a), the PC3 tumors stably expressing 15-LOX2sv-b were

also significantly smaller than the control tumors (Fig. 8, *b* and *c*). These results together indicate that restored expression of 15-LOX2 inhibits PCa cell proliferation *in vitro* and tumor development *in vivo* by functioning as a negative cell-cycle regulator. Like 15-LOX2, 15-LOX2sv-b also exhibits inhibitory effects.

DISCUSSION

The present study has made the following novel findings: 1) 15-LOX2 is expressed at multiple subcellular locations, including the cell-cell border and nucleus in addition to cytosol; 2) none of the three 15-LOX2 splice variants is expressed in the nucleus; 3) a putative NLS found in the N terminus of 15-LOX2 is partially involved in its nuclear targeting; 4) stable restoration of 15-LOX2 expression in PCa cells inhibits their proliferation *in vitro* and tumor development *in vivo*; and 5) 15-LOX2sv-b, which does not possess the AA-metabolizing activity and is mostly excluded from the nucleus, demonstrates similar inhibitory effects when overexpressed.

Localization of 15-LOX2 at the Cell-Cell Borders—A portion of 15-LOX2 is concentrated at the cell-cell borders in NHP cells *in vitro* as well as in prostate epithelial cells *in vivo* (Fig. 1). Located at the cell-cell borders are cell junctions, including occluding, anchoring, and communicating junctions (22). The anchoring junctions at the cell-cell borders mainly have two types: adherens junctions and desmosomes, both of which hold cells together and are formed by transmembrane adhesion proteins that belong to the cadherin family (22, 23). In adherens junctions, the cytoplasmic tails of cadherins (mainly E-cadherin) bind to anchor proteins (catenins, α-actinin, and vinculin) that tie them to actin filaments (22, 23). In desmosomes, the cytoplasmic tails of cadherins (desmoglein and desmocollin) bind to anchor proteins (plakoglobin and desmoplakin) that tie them to intermediate filaments keratins (22, 23). Interestingly, 15-LOX2 expressed at the cell-cell borders co-localizes with E-cadherin (Fig. 1), the major cadherin molecule expressed in epithelial cells. Western blotting analysis suggests that the 15-LOX2 expression pattern in multiple NHP strains and PCa cell lines coincides with that of a novel E-cadherin splice isoform: both are abundantly expressed in all primary strains and both are lost in all PCa cell lines examined (3).[2] Subcellular fractionation studies indicate that a significant portion of 15-LOX2 localizes to the CAP as well as the cytoskeleton and membrane fractions (Fig. 2). Together, these observations suggest that some 15-LOX2 molecules are probably associated with the E-cadherin-based adherens junctional structures that help maintain the prostate epithelial integrity. A provocative piece of evidence that supports this possibility is that both 15-LOX2 and E-cadherin are down-regulated or lost in PCa cells, and, in both cases, the loss of 15-LOX2 or E-cadherin expression is inversely correlated with grades and stages of the disease (4, 5, 24, 25).

Several other mammalian LOXs have also been shown to be localized in non-cytosolic compartments and interact with some of their constituents. For example, 5-LOX has been reported to bind actin and α-actinin (26). Platelet-type 12-LOX has been shown to be distributed in the membrane fractions (27) and may interact with some cytoskeletal proteins such as keratin and lamin (28). Finally, 15-LOX1 is well known to interact with, oxidize, and degrade intracellular organelle (*e.g.* ER and mitochondria) membranes (29, 30). These observations together suggest that LOX in general and 15-LOX2 in particular are localized at multiple subcellular microdomains and may participate in distinct cellular processes.

Nuclear Localization of 15-LOX2—Another particularly in-

teresting subcellular localization of 15-LOX2 is in the nucleus. Conceptually, this might provide an explanation to a conundrum we briefly touched upon before (3): how may 15-LOX2 inhibit cell-cycle progression? The main 15-LOX2 metabolite, 15(*S*)-HETE, has been shown to be a ligand for peroxisome proliferator-activated receptor γ or PPARγ (21, 31, 32), which has recently been shown to mediate cell-cycle arrest in a diverse array of cell types by suppressing cyclin D1 expression (33–35). Therefore, it is possible that 15-LOX2 may affect cell-cycle arrest in NHP cells (3) by activating PPARγ. However, the concentration of 15(*S*)-HETE required to activate PPARγ is generally ≥30 μM (21, 31, 32), which may be difficult to attain intracellularly. Therefore, the nuclear localization of 15-LOX2 may allow the generation of sufficient concentrations of the 15(*S*)-HETE ligand in the proximity of PPARγ to achieve activation of the receptor.

How is 15-LOX2 imported to the nucleus? A database search allowed us to identify a potential bipartite NLS at the N terminus of 15-LOX2. Site-specific mutagenesis studies reveal that this sequence is only partially involved in the nuclear import of 15-LOX2, because its mutations do not completely eliminate the nuclear expression of the molecule. This result is not surprising because many of these putative NLSs are not the sole determinants of or may even not be involved at all in protein nuclear import (36). The relevant example is 5-LOX, which translocates to the nucleus upon cell stimulation. Several groups identified a typical bipartite NLS ([638]RKNLEAIVS-VIAERNKKK[655]) that appears to be sufficient for 5-LOX nuclear localization (37–40), whereas another group found that the nuclear import of 5-LOX is probably mediated by a nonconventional signal located in the N-terminal β-barrel domain (41, 42). However, a recent study (43), using more rigorous structural and functional criteria, convincingly demonstrated that neither of these two sequences functions as the true NLS for 5-LOX. It turns out that most of the site-specific mutations (*e.g.* R651Q) carried out in these regions that eliminate the 5-LOX nuclear localization also abrogate the enzymatic activity of the protein, which seems to be important for the nuclear import (43). Instead, a previously unrecognized basic region, [518]RGRKSSGFPKSVK[530] located on a random coil of the catalytic domain, appears to function as the authentic NLS, because this sequence is sufficient to drive GFP to the nucleus and mutations of the underlined basic amino acids significantly diminish the nuclear import of 5-LOX without affecting the enzymatic activity (43). A homology search did not identify related sequence(s) in 15-LOX2. Therefore, it is still unclear how 15-LOX2 is imported into the nucleus. Perhaps the Arg[203]–Arg[221] NLS in 15-LOX2 cooperates with some other sequences or motifs to import the molecule to the nucleus.

Consistent with the notion that the Arg[203]–Arg[221] NLS is not the sole determinant of the 15-LOX2 nuclear localization, the three 15-LOX2 splice variants, which all retain this NLS, are mostly excluded from the nucleus. Because these splice variants do not share conserved regions in the sequences divergent from the parental 15-LOX2 (3), it is unlikely that their inability to go into the nucleus is due to deletion of an NLS in these variant-unique regions. The nuclear exclusion of these 15-LOX2 splice variants is also unlikely due to an overall reduced protein expression, because we have consistently observed similar mRNA (Fig. 6) as well as comparable protein expression levels (Figs. 3–5, and 7*b*) of 15-LOX2 and its variants or NLS mutants. It is possible that changes in protein folding or conformation somehow mask the responsible NLS and preclude these splice variants from interacting with importins, proteins required for nuclear import (19), and thus prevent their import. In support of this possibility, we have consistently noticed that

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[2] B. Bhatia and S. Tang, unpublished observations.

the anti-15-LOX2 antibody does not recognize well the denatured 15-LOX2 splice variants on Western blotting (Fig. 7, *a* and *c*; data not shown), suggesting that 15-LOX2 splice variants probably adopt different conformations from 15-LOX2. Alternatively, the reduced or lost enzymatic activity (*i.e.* to metabolize AA to produce 15(*S*)-HETE) renders these variants cytoplasmic, because it has been previously demonstrated that mutations that eliminate the 5-LOX enzymatic activity also abolish its nuclear import (see discussion above). Indeed, compared with 15-LOX2, 15-LOX2sv-a has decreased specificity and activity (2), whereas 15-LOX2sv-b is inactive (Table I). 15-LOX2sv-c is also predicted to be enzymatically dead, because this splice variant lacks the C-terminal isoleucine, which is conserved in all known LOXs and is required for the coordination of catalytic iron (44). Yet another possibility is that 15-LOX2, upon entering the nucleus, is retained in the organelle by physically interacting with one or more other proteins. The 15-LOX2 splice variants, on the other hand, due to structural changes, cannot be retained in the nucleus, although they might be able to be imported. We are currently exploring these possibilities.

15-LOX2sv-b Also Inhibits PCa Cell Proliferation and Tumor Development in Vivo—15-LOX2 is a negative cell-cycle regulator (3) and its expression is down-regulated or lost in PCa cells (3–6), suggesting that it may represent an endogenous prostate tumor suppressor. To lend direct support to this possibility, stable re-expression of 15-LOX2 in PCa cells inhibits their proliferation *in vitro* as well as tumor growth *in vivo*. Surprisingly, 15-LOX2sv-b, a splice variant that does not localize in the nucleus and does not possess AA-metabolizing enzymatic activity, also inhibits PCa cell proliferation and tumor growth. This observation is slightly different from our previous transient transfection experiments in which we found apparent but statistically insignificant inhibitory effect of 15-LOX2sv-b on PCa cell proliferation (3). A likely explanation for this discrepancy is that the inhibitory effect of 15-LOX2sv-b is manifested more slowly than that of 15-LOX2 so that by 48 h after transfection only a small inhibitory effect was observed for 15-LOX2sv-b (3). Therefore, the inhibitory effect of 15-LOX2sv-b is fully manifested in the stable clones (this study). Another possibility is that, in previous transient transfection experiments, we used the pCMS expression constructs in which 15-LOX2sv-b and GFP were driven by separate promoters (3). As pointed out under "Results," in some cells transfected with the pCMS expression constructs the 15-LOX2sv-b (and 15-LOX2) expression is preferentially lost, which may lead to an underestimation of their inhibitory effect on PCa cell proliferation. On the other hand, a tumor-suppressive function of 15-LOX2sv-b is consistent with our previous findings that the mRNA and protein levels of 15-LOX2 splice variants are also reduced in multiple PCa cells (3). The precise biological roles of 15-LOX2 as well as various 15-LOX2 splice variants, the latter of which are also expressed *in vivo*,[3] in maintaining physiological prostate homeostasis and in PCa development remain to be clarified. Nevertheless, the results presented in this study raise the possibility that 15-LOX2 may possess biological activities independent of AA-metabolizing activity and independent of its nuclear localization. How 15-LOX2 inhibits PCa cell proliferation without resorting to AA metabolism is currently unclear. One possibility is that 15-LOX2 as well as its splice variants might directly catalyze the oxidation and degradation of biomembranes, analogous to 15-LOX1 (29, 30).

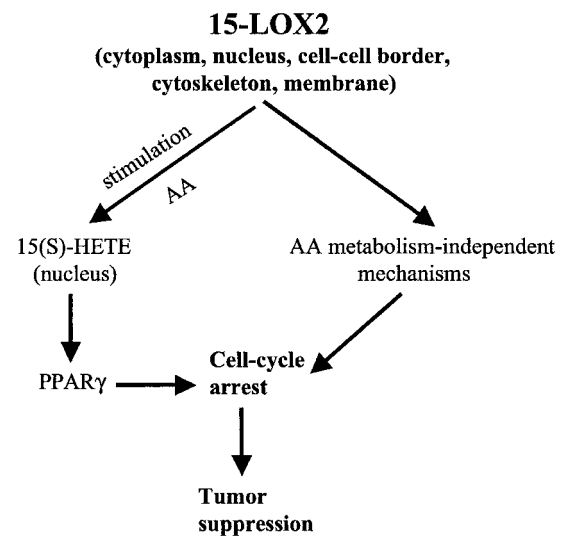Together, the data presented herein suggest at least two signaling pathways that could conceptually mediate the biological functions of 15-LOX2 (Fig. 9). Under physiological, unstimulated conditions, 15-LOX2 as well as its splice variants may inhibit cell proliferation through as-yet-unknown mechanisms independent of the nuclear localization and enzymatic activity (*i.e.* AA metabolism). Furthermore, localization of 15-LOX2 to the cell-cell borders and its association with the cytoskeleton may help maintain the differentiated phenotype of prostate glands. Under stimulated conditions, AA will be mobilized resulting in increased 15(*S*)-HETE production in the cells, especially in the nucleus, which may lead to PPARγ-dependent cell-cycle arrest. This model explains why PCa cells suppress the expression of both 15-LOX2 and its enzymatically inactive splice variants (3). The model also predicts that restoration of 15-LOX2 or its splice variant expression should suppress PCa development, a prediction borne out by orthotopic tumor implantation analysis (Fig. 8).

Fig. 9. **A hypothetical model of the mechanisms of action of 15-LOX2.** See text for details.

REFERENCES

1. Brash, A. R., Boeglin, W. E., and Chang, M. S. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 6148–6152
2. Kilty, I., Alison, L., and Vickers, P. J. (1999) *Eur. J. Biochem.* **266,** 83–93
3. Tang, S., Bhatia, B., Maldonado, C., Yang, P., Newman, R. A., Liu, J., Chandra, D., Traag, J., Klein, R. D., Fischer, S. M., Chopra, D., Shen, J., Zhau, H., Chung, L. W. K., and Tang, D. G. (2002) *J. Biol. Chem.* **277,** 16189–16201
4. Shappell, S. B., Boeglin, W. E., Olson, S. J., Kasper, S., and Brash, A. R. (1999) *Am. J. Pathol.* **155,** 235–245
5. Jack, G. S., Brash, A. R., Olson, S. J., Manning, S., Coffey, C. S., Smith, J. A., Jr., and Shappell, S. B. (2000) *Hum. Pathol.* **31,** 1146–1154
6. Shappell, S. B., Manning, S., Boeglin, W. E., Guan, Y. F., Roberts, R. L., Davis, L., Olson, S. J., Jack, G. S., Coffey, C. S., Wheeler, T. M., Breyer, M. D., Brash, A. R. (2001) *Neoplasia* **3,** 287–303
7. Chopra, D. P., Grignon, D. J., Joiakim, A., Mathieu, P. A., Mohamed, A., Sakr, W. A., Powell, I. J., and Sarkar, F. H. (1996) *J. Cell. Physiol.* **169,** 269–280
8. Chopra, D. P., Sarkar, F. H., Grignon, D. J., Sakr, W. A., Mohamed, A., and Waghray, A. (1997) *Cancer Res.* **57,** 3688–3692
9. Tang, D. G., Li, L., Chopra, D., and Porter, A. T. (1998) *Cancer Res.* **58,** 3466–3479
10. Tang, D. G., Timar, J., Grossi, I. M., Renaud, C., Kimler, V., Diglio, C. A., Taylor, J. D., Honn, K. V. (1993) *Exp. Cell Res.* **207,** 361–375
11. Joshi, B., Li, L., Taffe, B. G., Zhu, Z., Ben-Josef, B., Taylor, J. D., Porter, A. T., and Tang, D. G. (1999) *Cancer Res.* **59,** 4343–4355
12. Liu, J.-W., Chandra, D., Tang, S.-H., Chopra, D., and Tang, D. G. (2002) *Cancer Res.* **62,** 2976–2981
13. Chandra, D., Liu, J.-W., and Tang, D. G. (2002) *J. Biol. Chem.* **277,** 50842–50854
14. Li, L., Zhu, Z., Joshi, B., Zhang, C., Johnson, C. R., Marnett, L. J., Honn, K. V., Crissman, J. D., Porter, A. T., and Tang, D. G. (1999) *Anticancer Res.* **19,** 61–70
15. Tang, D. G., Chen, Y., Diglio, C. A., and Honn, K. V. (1993) *J. Cell Biol.* **121,** 689–704

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[3] S. B. Shappell, unpublished observations.

16. Evans, W. H. (1992) in *Preparative Centrifugation: A Practical Approach* (Rickwood, D., ed) pp. 233–270, The IRL Press, Oxford, UK
17. Scheffler, I. E. (1999) *Mitochondria*, Wiley-Liss, New York, pp. 26–33
18. Breckenridge, D. G., Nguyen, M., Kuppig, S., Reth, M., and Shore, G. C. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 4331–4336
19. Kaffman, A., and O'Shea, E. (1999) *Annu. Rev. Cell Dev. Biol.* **15,** 291–339
20. Cokol, M., Nair, R., and Rost, B. (2000) *EMBO Rep.* **1,** 411–415
21. Shappell, S. B., Gupta, R. A., Manning, S., Whitehead, R., Boeglin, W. E., Schneider, C., Case, T., Price, J., Jack, G. S., Wheeler, T. M., Matusik, R. J., Brash, A. R., and DuBois, R. N. (2001) *Cancer Res.* **61,** 497–503
22. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002) *Molecular Biology of the Cell*, 4th Ed., pp. 1065–1090, Garland Science, Taylor & Francis Group, New York
23. Takeichi, M. (1991) *Science* **251,** 1451–1455
24. Umbas, R., Schalken, J. A., Aalders, T. W., Karthaus, H. F., Schaafsman, H. E., Debruyne, F. M., and Isaacs, W. B. (1992) *Cancer Res.* **52,** 5104–5109
25. Bussemakers, M. J., Van Bokhoven, A., Tomita, K., Jansen, C. F., and Schalken, J. A. (2000) *Int. J. Cancer* **85,** 446–450
26. Lepley, R. A., and Fitzpatrick, F. A. (1984) *J. Biol. Chem.* **269,** 24163–24168
27. Timar, J., Raso, E., Dome, B., Li, L., Grignon, D., Nie, D., Honn, K. V., and Hagmann, W. (2000) *Int. J. Cancer* **87,** 37–43
28. Tang, K., Finley, R. L., Jr., Nie, D., and Honn, K. V. (2000) *Biochemistry* **39,** 3185–3191
29. Kuhn, H., and Borngraber, S. (1999) *Adv. Exp. Med. Biol.* **447,** 5–28
30. Walther, M., Anton, M., Wiedmann, M., Fletterick, R., and Kuhn, H. (2002) *J. Biol. Chem.* **277,** 27360–27366
31. Kersten, S., Desvergne, B., and Wahli, W. (2000) *Nature* **405,** 421–424
32. Huang, J. T., Welch, J. S., Ricote, M., Binder, C. J., Wilson, T. M., Kelly, C., Witztum, J. L., Funk, C. D., Conrad, D., and Glass, C. K. (1999) *Nature* **400,** 378–382
33. Wang, C., Fu, M., D'Amico, M., Albanese, C., Zhou, J.-N., Brownlee, M., Lisanti, M. P., Chatterjee, V. K. K., Lazar, M. A., and Pestell, R. G. (2001) *Mol. Cell. Biol.* **21,** 3057–3070
34. Wakino, S., Kintscher, U., Kim, S., Yin, F., Hsueh, W. A., and Law, R. E. (2000) *J. Biol. Chem.* **275,** 22435–22441
35. Kitamura, S., Miyazaki, Y., Hiraoka, S., Nagasawa, Y., Toyota, M., Takakra, R., Kiyohara, T., Shinomura, Y., and Matsuzawa, Y. (2001) *Int. J. Cancer* **94,** 335–342
36. Dingwall, C., and Laskey, R. A. (1991) *Trends Biochem. Sci.* **16,** 478–481
37. Lepley, R. A., and Fitzpatrick, F. A. (1998) *Arch. Biochem. Biophys.* **356,** 71–76
38. Healy, A. M., Peters-Golden, M., Yao, J. P., and Brock, T. G. (1999) *J. Biol. Chem.* **274,** 29812–29818
39. Hanaka, H., Shimizu, T., and Izumi, T. (2002) *Biochem. J.* **361,** 505–514
40. Christmas, P., Fox, J. W., Ursino, S. R., and Soberman, R. J. (1999) *J. Biol. Chem.* **274,** 25594–25598
41. Chen, X. S., Zhang, Y.-Y., and Funk, C. D. (1998) *J. Biol. Chem.* **273,** 31237–31244
42. Chen, X.-S., and Funk, C. D. (2001) *J. Biol. Chem.* **276,** 811–818
43. Jones, S. M., Luo, M., Healy, A. M., Peters-Golden, M., and Brock, T. G. (2002) *J. Biol. Chem.* **277,** 38550–38556
44. Brash, A. R. (1999) *J. Biol. Chem.* **274,** 23679–23682

Evidence that Sp1 positively and Sp3 negatively regulate and androgen does not directly regulate functional tumor suppressor 15-lipoxygenase 2 (15-LOX2) gene expression in normal human prostate epithelial cells

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In this project, we studied the gene regulation of 15-lipoxygenase 2 (15-LOX2), the most abundant arachidonate-metabolizing LOX in adult human prostate and a negative cell-cycle regulator in normal human prostate (NHP) epithelial cells. Through detailed *in silico* promoter examination and promoter deletion and activity analysis, we found that several Sp1 sites (i.e., three GC boxes and one CACCC box) in the proximal promoter region play a critical role in regulating 15-LOX2 expression in NHP cells. Several pieces of evidence further suggest that the Sp1 and Sp3 proteins play a physiologically important role in positively and negatively regulating the 15-LOX2 gene expression, respectively. First, mutations in the GC boxes affected the 15-LOX2 promoter activity. Second, both Sp1 and Sp3 proteins were detected in the protein complexes that bound the GC boxes revealed by electrophoretic mobility shift assay. Third, importantly, inhibition of Sp1 activity or overexpression of Sp3 both inhibited the endogenous 15-LOX2 mRNA expression. Since 15-LOX2 is normally expressed in the prostate luminal epithelial cells, we subsequently explored whether androgen/androgen receptor may directly regulate its gene expression. The results indicate that androgen does not directly regulate 15-LOX2 gene expression. Together, these observations provide insight on how 15-LOX2 gene expression may be regulated in NHP cells.

Introduction

15-Lipoxygenase 2 (15-LOX2) shows the highest homology to murine 8-LOX, has at least three splice variants (termed 15-LOX2sv-a/b/c (15-LOX2 splice variant a, b or c)), mainly metabolizes arachidonic acid (AA) to 15(S)-hydroxyeicosatetraenoic acid [15(S)-HETE], and is primarily expressed in prostate, lung, skin, and cornea (Brash *et al*., 1997; Kilty *et al*., 1999; Tang *et al*., 2002). 15-LOX2 expression and activity are decreased in high-grade prostate intraepithelial neoplasia and prostate cancer (PCa) (Shappell *et al*., 1999). We recently reported that 15-LOX2 is a negative cell-cycle regulator in normal human prostate (NHP) epithelial cells (Tang *et al*., 2002), which may explain why it is advantageous for PCa cells to suppress its expression. Not surprisingly, re-expression of 15-LOX2 inhibits PCa cell proliferation *in vitro* and tumor development *in vivo* (Bhatia *et al*., 2003), suggesting that 15-LOX2 may represent a functional prostate tumor suppressor. Surprisingly, however, the tumor-suppressive function of 15-LOX2 does not appear to absolutely require its localization to the nucleus or its ability to metabolize AA, as 15-LOX2sv-b, a splice variant that does not localize to the nucleus and lacks obvious AA-metabolizing activity also demonstrates tumor-inhibitory effect (Bhatia *et al*., 2003).

To fully understand the role of 15-LOX2 in regulating prostate development and homeostasis and the contribution of its loss of expression to PCa development, we must first understand how the gene is regulated in NHP cells, which is the main goal of the current study. By utilizing a variety of cell biological, biochemical, and molecular approaches, we provide evidence that the Sp1 and Sp3 transcription factors positively and negatively regulate 15-LOX2 gene expression in NHP cells. In contrast, androgen/androgen receptor (AR) pathway does not directly regulate the 15-LOX2 gene expression.

Results

Determination of TSS and analysis of the putative 15-LOX2 promoter

We first cloned out a ∼1.2 kb 5′-flanking region (i.e. the P3–P8 fragment; Table 1) immediately upstream of

ATG (Figure 1b). Using this fragment, we performed a primer extension analysis to pinpoint the TSS of the 15-LOX2 gene. As shown in Figure 1a, the TSS of 15-LOX2 was identified as an adenine in the sequence CAA TA*A*CCA, 87 bp upstream of ATG (Figure 1b and c).
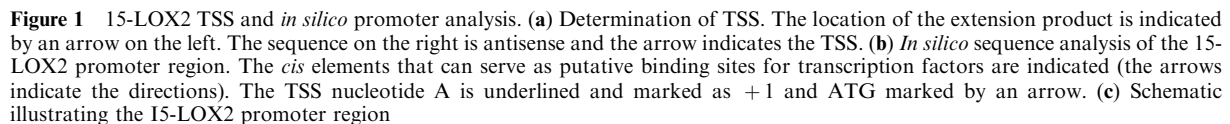
Using the determined TSS, we performed *in silico* sequence analysis on 15-LOX2 promotor (Figure 1b), using TESS at www.cbil.upenn.edu/tess, TFSEARCH at www.cbrc.jp/research/db/TFSEARCH.html, Cister at http://zlab.bu.edu/˜mfrith/cister.shtml, PROMO at www.lsi.upc.es/˜alggen/recerca/promo_v2/ frame-promo.html, and Gene Regulation at www.gene-regulation.com, as well as searching several transcription factor compilations (Locker, 1993; Strachan and Read, 2000) and databases (e.g. TRANSFAC, TRANSCompel, and IMD) (Heinemeyer *et al*., 1998; Matys *et al*., 2000; Frith *et al*., 2001). This analysis revealed some interesting features for the 15-LOX2 promoter region (Figure 1a and c). First, the 15-LOX2 promoter does not have a TATA (consensus TATAAA) or TATA-like (TATTT) box, suggesting that 15-LOX2 is a tissue-specific 'housekeeping' gene. Second, most TATA-less promoters utilize the so-called initiator element (Inr; consensus PyA(A/T)PyPy, where Py is a pyrimdine) and/or downstream promoter element (DPE; consensus (AG)G(AT)CGTG) located at various distances downstream of the TSS to initiate transcription (Strachan and Read, 2000; Levine and Tijan, 2003). The 15-LOX2 TSS (CAATA*A*CC) conformed to the consensus Inr sequence and a DPE with sequence AGGCGTG that matches the consensus DPE sequence was found 16 bp downstream of the TSS (Figure 1b and c), suggesting that 15-LOX2 gene transcription might utilize these elements. Third, multiple potentially important transcription factor-binding sites were concentrated in the 15-LOX2 proximal promoter region (i.e. within ∼120 bp region upstream of TSS). For example, a CAAT box (consensus (A/G)CCAATC) with the sequence GCCAATC was found at position −114 (Figure 1b and c). This *cis* element generally serves as

Table 1 Primers and probes used in the current study

Name	Position[a]	Orientation	Sequence
PCR primers			
P2	−3985/−3957	Forward	5′-GTCCTTGGAGGTGCAAGATCACAGGTT-3′
P3	−1116/−1093	Forward	5′-CCTTCCCACCTCTGCTTCTCACTC-3′
P4	−726/−706	Forward	5′-CAATAGCAGTGACGATAGCAC-3'
P5	−471/−452	Forward	5′-GGCTGAGGTAGGAGAATCAC-3′
P6	−163/−149	Forward	5′-CCCAAACCTCAGGGT-3′
P7	−102/−83	Forward	5′-CCCACTTTAGTTGCGTGTTC-3′
P8	+60/+80	Reverse	5′-GCCTAAGTCCAGCTCTCTACG-3′
P9	−151/−126	Reverse	5′-TGGGAACAGGAGGGACTAAGAATAC-3′
P10	+227/+250	Reverse	5′-CCCCATCCACTCCCACGCACTCA-3′
Site-specific mutagenesis primers[b]			
GC box 1/CACCC box		CACCC box	
Wild type			5′-CAGCGCTGCCAATCCCCCGCCCACCCCCACTTTAGTTGC-3′
Mutant			5′-CAGCGCTGCCAATC<u>CCCCG</u>***TCTAGA***CCCACTTTAGTTGC-3′
			*Xba*I
GC box 2[c]			
Wild type			5′-CCAGCCTCTCCGCCCCGCCCCTCCCCGCCCTG-3′
Mutant			5′-CCAGCCTCTCCG<u>CC</u>***AAGCTT***CT<u>CCCCGCCCTG</u>-3′
			*Hind*III
GC box 3[d]			
Wild type			5′-CTCCG<u>CCCCGCCCCT</u>CCCCGCCCTGAAACGGACGTG-3′
Mutant			5′-CTCCG<u>CCCCGCCCCTC</u>***AACGCT***CTGAAACGGACGTG-3′
Double-strand oligonucleotide probes used in EMSA[b]			
Consensus Sp1 probe (Santa Cruz)			
Wild type			5′-ATTCGATCGGGGCGGGGCGAG-3′
Mutant			5′-ATTCGATC<u>GG***TT***CGGGGC</u>GAG-3′
GC1 probe		CAAT box CACCC box	
Wild type			5′-CAGCGCTG<u>CCAATC</u>CCCCGCCC<u>ACCCCC</u>ACTTTAGTTGC-3′
Mutant			5′-CAGCGCTGCCAATC<u>CCCCG</u>***TCTAGA***CCCACTTTAGTTGC-3′
GC2/GC3 probe			
Wild type			5′-CTCCG*CCCCGCCCC*T*CCCCGCCC*TGAAACG-3′
GC2 mutant[c]			5′-CTCCG<u>CC***AAGCTT***CT</u>CCCCGCCCTGAAACG-3′
GC3 mutant[d]			5′-CTCCG<u>CCCCGCCCCTC</u>***AACGCT***CTGAAACG-3′

[a]Relative to TSS, which is designated as +1. The overlapping GC box 1 and CACCC box are located at −113/−104 and the tandem GC box 2 and GC box 3 at −34/−53. See Figure 1 and text for details. [b]For the sake of simplicity, only the upper strand sequence is shown. GC boxes are underlined and CAAT and CACCC boxes are indicated on top. Mutated sequences are highlighted in italic. [c]In this mutant, GC box 3 remains intact (indicated by the second underlined sequence in wild type). [d]In this mutant, GC box 2 remains intact (indicated by the first underlined sequence in wild type)

Figure 1 15-LOX2 TSS and *in silico* promoter analysis. (**a**) Determination of TSS. The location of the extension product is indicated by an arrow on the left. The sequence on the right is antisense and the arrow indicates the TSS. (**b**) *In silico* sequence analysis of the 15-LOX2 promoter region. The *cis* elements that can serve as putative binding sites for transcription factors are indicated (the arrows indicate the directions). The TSS nucleotide A is underlined and marked as +1 and ATG marked by an arrow. (**c**) Schematic illustrating the I5-LOX2 promoter region

the binding site for ubiquitous transcription factors NF-1 (also called CTF for *C*CAAT-binding *t*ranscription *f*actor), NF-Y (also called CBF for *C*CAAT box-binding *f*actor), and C/EBP (Strachan and Read, 2000). Most prominently, a CACCC box with the sequence CCACCCC that matches the consensus and three GC boxes (consensus GGGGCGGGG) were found within ~120 bp upstream of the TSS (Figure 1b,c). CACCC box, GC box, and some other related GC-rich sequences are frequently called Sp1 sites, which generally are located at −160/+1 region and serve as binding sites for the Sp/KLF (Krüppel-like factors) transcription factors to modulate the basal and induced transcription of the core promoter as well as operate as essential enhancer sequences (Locker, 1993; Strachan and Read, 2000; Black *et al*., 2001). Fourth, multiple other perfectly matched transcription factor-binding sites including three GATA sites, three Nkx2.5 sites, one CdxA-binding site, one MBF-1 site, one silencer-1 site, two AP2 sites, and one SRY box were also found upstream of the TSS (Figure 1b). A stretch of six repeats containing sequence AAAT was present just upstream of the MBF-1 site (Figure 1b; boxed sequence). The significance of this interesting sequence feature remains unclear. Finally, examination of the 1.2 kb 15-LOX2 promoter region did not reveal androgen-responsive element (ARE), although a perfect estrogen-responsive

element was found at ~500 bp upstream of the TSS (Figure 1b).

Sp1 sites as crucial cis elements regulating the 15-LOX2 promoter activity

Next, we carried out deletion analysis of the 1.2 kb 15-LOX2 promoter. The −1116/+80 (i.e. P3–P8; Table 1) fragment possessed 44-fold higher promoter activity than the vector (pGL3-basic) alone (not shown). The −726/+80 fragment demonstrated ~80% of the −1116/+80 promoter activity (Figure 2a). In contrast, the −471/+80 fragment showed a slightly higher promoter activity (Figure 2a), suggesting the presence of a potential negative regulatory element within the −726/−471 region. Notably, the −163/+80 fragment, which contained all four Sp1 sites, had nearly the full promoter activity as the −1116/+80 fragment, whereas the −102/+80 fragment, which contained only GC box 2 and GC box 3, demonstrated ~70% of the −1116/+80 promoter activity (Figure 2a). In contrast, the −726/−126 and −471/−126 fragments, both of which lack all four Sp1 sites (Figure 1b; Table 1), showed no promoter activity (Figure 2a).

In another set of experiments using a different batch of NHP6 cells (Figure 2b), we utilized the −726/+80 fragment as the baseline, which possessed 36-fold higher
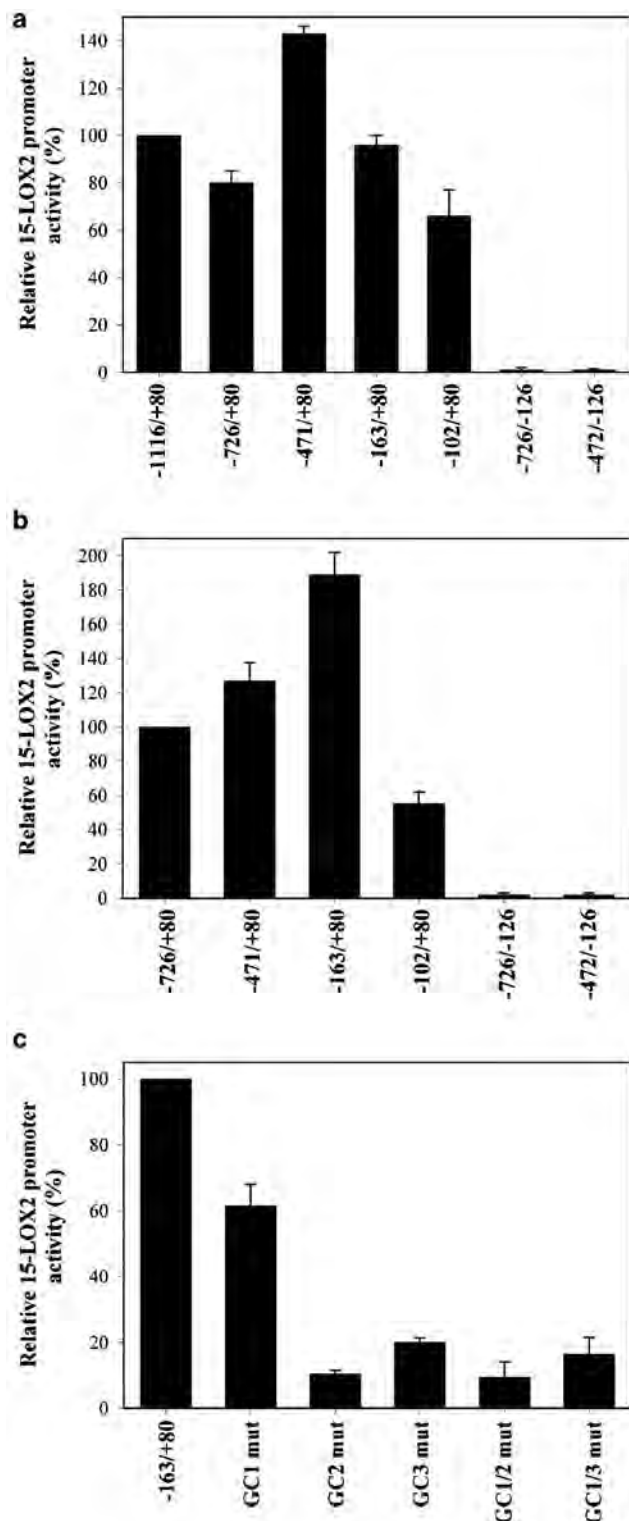
Figure 2 Critical roles of GC boxes. (**a**) Luciferase assays ($n = 3$) performed using NHP6 cells (P5–7) and the relative promoter activities presented as the % of the $-1116/+80$ fragment. (**b**) Luciferase assays ($n = 6$) performed using NHP6 cells (P6-7) and the relative promoter activities presented as the % of the $-726/+80$ fragment. Data in both (**a**) and (**b**) represent the mean ± s.e.m. (**c**) Site-specific mutagenesis. Luciferase constructs were transfected into NHP6 cells (P6). The relative 15-LOX2 promoter activities were expressed as the % of the $(-163/+80)$-luc, which had a RLU of 44 over the pGL3-basic vector. The data represent the mean ± s.e.m. ($n = 3$)

promoter activity than the vector alone (not shown). Interestingly, the $-471/+80$ fragment had slightly higher promoter activity and the $-163/+80$ fragment consistently showed a ~ 2-fold increase in the promoter activity compared to the $-726/+80$ fragment (Figure 2b), suggesting an inhibitory *cis* element(s) between $-471/-163$. Again, the $-102/+80$ fragment demonstrated $\sim 60\%$ of the $-726/+80$ promoter activity, whereas both $-726/-151$ and $-471/-151$ fragments completely lacked promoter activity (Figure 2b). Similar results were obtained in experiments carried out in NHP2 cells (not shown).

Crucial role of GC box 2 and GC box 3 in regulating the 15-LOX2 promoter activity

Next, we carried out site-specific mutagenesis (Table 1). As shown in Figure 2c, mutation of GC box 1/CACCC box (GC1 mut) eliminated $\sim 40\%$ of the promoter activity. In contrast, mutation of GC box 2 or GC box 3 eliminated 90 and 80% promoter activity, respectively (Figure 2c). Double mutations of GC box 1/CACCC box with GC box 2 or GC box 3 slightly reduced the promoter activity further (Figure 2c). These results together suggest that the GC box 2 and GC box 3 and, less significantly, GC1 box 1/CACCC box, play a crucial role in regulating the 15-LOX2 promoter activity.

Sp1 as a positive regulator of the 15-LOX2 promoter activity and gene expression

The above experiments suggest that the sequence in the proximal 15-LOX2 promoter region containing Sp1 sites is critical for the 15-LOX2 gene expression. Sp1 and related Sp family members such as Sp3 are the major transcription factors that bind to GC-rich Sp1 sites (Black *et al.*, 2001). Sp1 sites and the Sp family proteins have been implicated in the constitutive expression of many 'housekeeping' genes as well as in tissue and cell specific and highly regulated expression of many other genes (Huang *et al.*, 2000; Hong *et al.*, 2002; Blais *et al.*, 2002). Furthermore, Sp1 sites and the Sp1 protein have been implicated in regulating the basal expression of several other LOXs, including 12-LOX and 15-LOX 1 (Kritzik *et al.*, 1997; Kelavkar *et al.*, 1998; Chen and Chang, 2000).

To study the role of Sp1, we took advantage of *Drosophila melanogaster* Schneider SL2 cells, which lack endogenous Sp1 or other Sp family proteins (Black *et al.*, 2001). As shown in Figure 3a, cotransfection of the $(-726/+80)$-luc or $(-102/+80)$-luc with an Sp1 expression vector driven by *Drosophila* actin promoter (pPacSp1) into SL2 cells enhanced, in a dose-dependent manner, their promoter activities. As expected, the $-102/+80$ fragment showed $\sim 50\%$ luciferase activity compared to the $-726/+80$ fragment (Figure 3a), because the $-726/+80$ fragment contains four Sp1 sites, whereas the $-102/+80$ fragment only two Sp1 sites (Figure 1b). These results provide direct evidence that the Sp1 protein can activate the 15-LOX2 promoter.
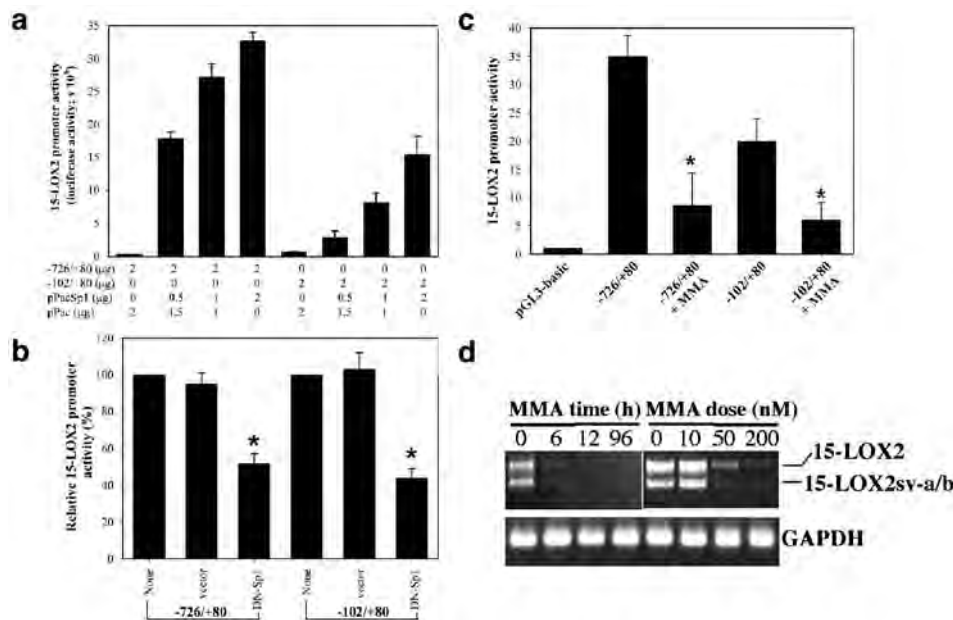
Figure 3 Sp1 as a positive regulator. (**a**) SL2 cells (2×10^5 cells/well) were transfected with the indicated plasmids at the respective doses (pPac was cotransfected to maintain equal plasmid amount). Cell lysates were made 48 h after transfection and equal amounts of proteins used for luciferase activity measurement. Results were expressed as RLU normalized to that obtained with the empty vector (pPac) alone. Data represent the mean $\pm$ s.e.m. ($n = 3$). $P < 0.01$ at all dose points (Student's t-test). (**b**) NHP6 cells (P6) were transfected with the promoter plasmids together with a DN-Sp1 vector (pEBG-Sp1) or its control vector (pEBG-N). At 48 h after transfection, cells were harvested for luciferase activity measurement. Data represent the mean $\pm$ s.e.m. derived ($n = 3$). *$P < 0.01$ (Student's t-test). (**c**) NHP6 cells (P5–6) were transfected with pGL3-basic control vector or promoter construct in the presence or absence of MMA (200 nM). After 48 h, cells were harvested for luciferase activity assays. The promoter activities were expressed as RLU (mean $\pm$ s.e.m.; $n = 3$). *$P < 0.01$ (Student's t-test). (**d**) NHP6 cells (P6) were treated with 200 nM MMA for various time intervals (time course) or with various doses of MMA for 48 h (dose study). RT–PCR was performed using primers C and D that could pick up both 15-LOX2 and 15-LOX2sv-a/b (Tang *et al.*, 2002)

To determine whether the Sp1 protein is required for 15-LOX2 gene expression, we cotransfected a DN-Sp1 expression vector together with the promoter constructs into NHP6 cells. The DN-Sp1 has no transactivating function and selectively inhibits Sp1-dependent reporter gene expression (Ptersohn and Thiel, 1996; Grinstein *et al.*, 2002). As shown in Figure 3b, DN-Sp1 at 0.5 μg/well inhibited the $-726/+80$ and $-102/+80$ promoter activities by 50–60%, respectively. At a higher concentration (i.e. 2 μg/ml), DN-Sp1 reduced both promoter activities to $\sim$30% (not shown). As an alternative approach, NHP6 cells were treated with an Sp1-specific chemical inhibitor, MMA (Kaluz *et al.*, 2003), simultaneously with the transfection of promoter fragments. As shown in Figure 3c, MMA significantly inhibited the promoter activities of both fragments. MMA inhibited not only the exogenous 15-LOX2 promoter activity but also the endogenous 15-LOX2 expression revealed by reverse transcriptase–polymerase chain reaction (RT—PCR) (Figure 3d). Time-course studies revealed that MMA (at 200 nM) nearly completely inhibited the expression of both 15-LOX2 and 15-LOX2sv-a/b as early as 6 h post-treatment (Figure 3d). Dose studies indicated that MMA at 50 nM inhibited 15-LOX2 mRNA expression in NHP6 cells by $>$90% at 48 h post-treatment (Figure 3d). Altogether, these results suggest that Sp1, through binding to the GC-rich sequences in the proximal 15-LOX2 promoter region,

functions as a positive regulator of 15-LOX2 gene expression. The data also establish that 15-LOX2 gene expression in NHP cells are Sp1 dependent.

Sp3 as a negative regulator of the 15-LOX2 promoter activity and gene expression

Among the Sp family proteins, Sp3 has been shown to either positively (Garcia-Ruiz *et al.*, 2002; Won *et al.*, 2002; Schafer *et al.*, 2003) or negatively (Hagen *et al.*, 1994; Kumar and Butler, 1997) modulate the Sp1-dependent gene expression. To determine whether and how Sp3 may modulate the Sp1-dependent 15-LOX2 expression, we again made use of SL2 cells. As shown in Figure 4a, Sp3 dose dependently inhibited Sp1-dependent 15-LOX2 promoter activity in SL2 cells. When Sp3 was transfected into NHP6 cells, it completely inhibited the 15-LOX2 promoter, that is, $(-163/+80)$-luc, activity (Figure 4b). Importantly, overexpression of Sp3 also reduced the mRNA levels of both 15-LOX2 and 15-LOX2sv-a/15-LOX2sv-b (Figure 4c). Considering that the transfection efficiency in NHP cells was generally $<$10% (Tang *et al.*, 2002), the inhibitory effect of Sp3 on endogenous 15-LOX2 mRNA expression was significant (Figure 4c). Interestingly, for unknown reasons, we consistently observed a shift of 15-LOX2sv-a/15-LOX2sv-b to 15-LOX2 upon transfection with the control vector (Figure 4c, the middle lane).
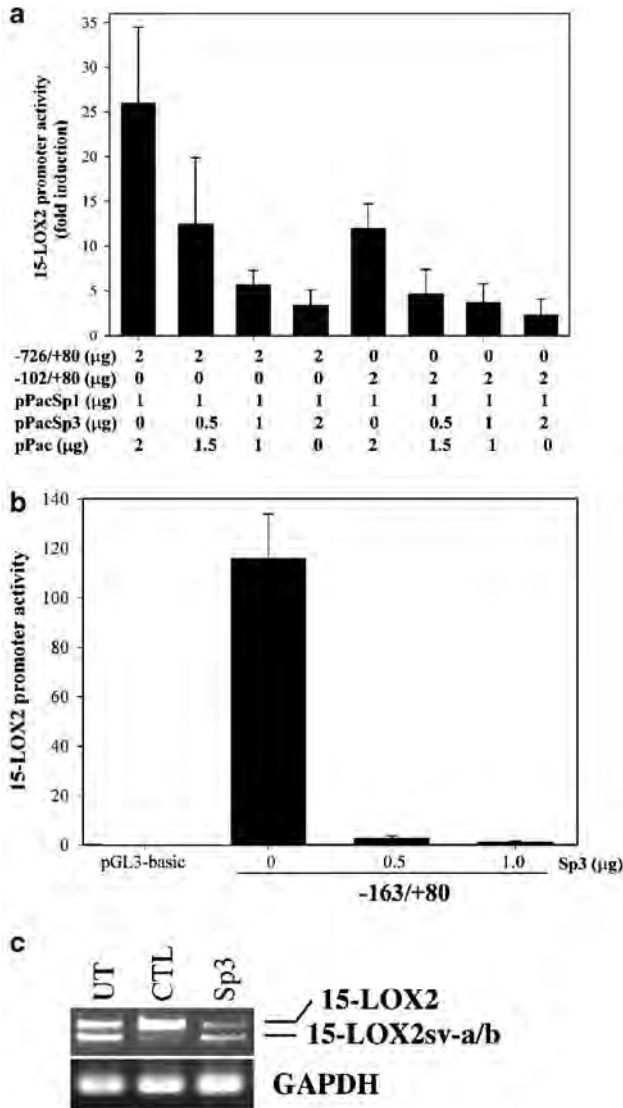
electrophoretic mobility shift assay (EMSA) experiments using labeled GC1 or GC2/GC3 probe (Table 1) and NE prepared from either NHP6 (Figure 5) or PCa (Figure 6) cells. There were at least four complexes, that is, complex I–IV (Figure 5a, lane 2) that bound to the GC box 1/CACCC box (Figure 1b; Table 1), as their binding could be completely competed out by cold GC1 probe (Figure 5a, lane 3). Note that a prominent band running faster than complex IV was not competed out by the cold GC1 probe (Figure 5a, lane 3) and perhaps represented a nonspecific band (Figure 5a; NS).
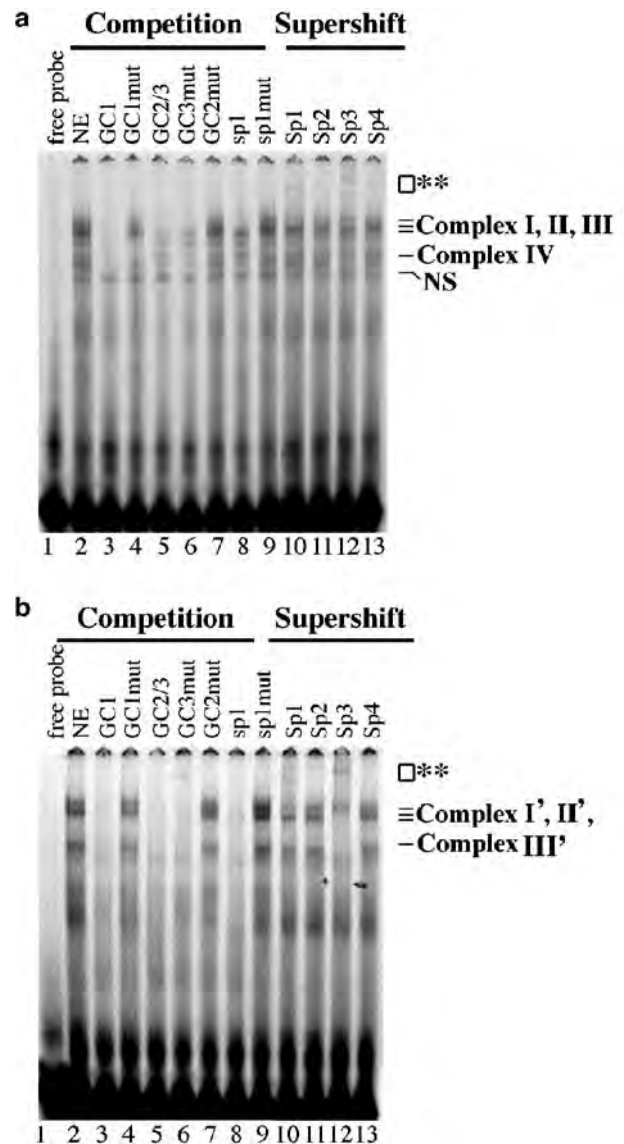

Figure 4 Sp3 as a negative regulator. (**a**) Experiments were performed as described in Figure 3a except for the addition of pPacSp3. The results are expressed as fold induction. Data represent the mean $\pm$ s.e.m. ($n = 3$). $P < 0.01$ at all dose points. (**b**) NHP6 cells (P7) were transfected with pGL3-basic or the ($-163/+80$)-luc in the presence of the control vector (i.e. pCMVneo; 0) or pCMV-Sp3. Luciferase assays were carried out 48 h after transfection. The 15-LOX2 promoter activity was expressed as RLU and data represent the mean $\pm$ s.e.m. ($n = 2$). Sp3 at both doses significantly ($P < 0.01$) inhibited the 15-LOX2 promoter activity. (**c**) Sp3 inhibits endogenous 15-LOX2 gene expression. NHP6 cells (P5) were either untransfected (UT) or transfected with pCMVneo (CTL) or pCMV-Sp3 (Sp3; 0.5 μg/well) for 48 h. RT–PCR was performed as in Figure 3d

These results, altogether, suggest that Sp3 negatively regulates the 15-LOX2 gene expression in NHP cells.

Multiple complexes form in the GC-rich regions of the 15-LOX2 promoter: Sp1 and Sp3, but not Sp2 or Sp4, exist in some of these complexes

To determine directly whether Sp1 and Sp3 interact with the GC boxes of the 15-LOX2 promoter, we carried out

Figure 5 EMSA in NHP6 cells. (**a**) EMSA using GC1 probe and NE from NHP6 cells (P6). Two types of experiments, that is, competition and supershift, were performed. The unlabeled competition probes (either wild-type or mutant) and the supershifting antibodies used were indicated on the top. Distinct complexes were indicated on the right. Arrows, the supershifted bands. NE, nuclear extract; NS, nonspecific. (**b**) EMSA using GC2/GC3 probe. Legend is similar to that described in (**a**). In both (**a**) and (**b**), asterisks indicate the supershifted bands. Autoradiograms shown were representative of three to four repeat experiments with comparable results
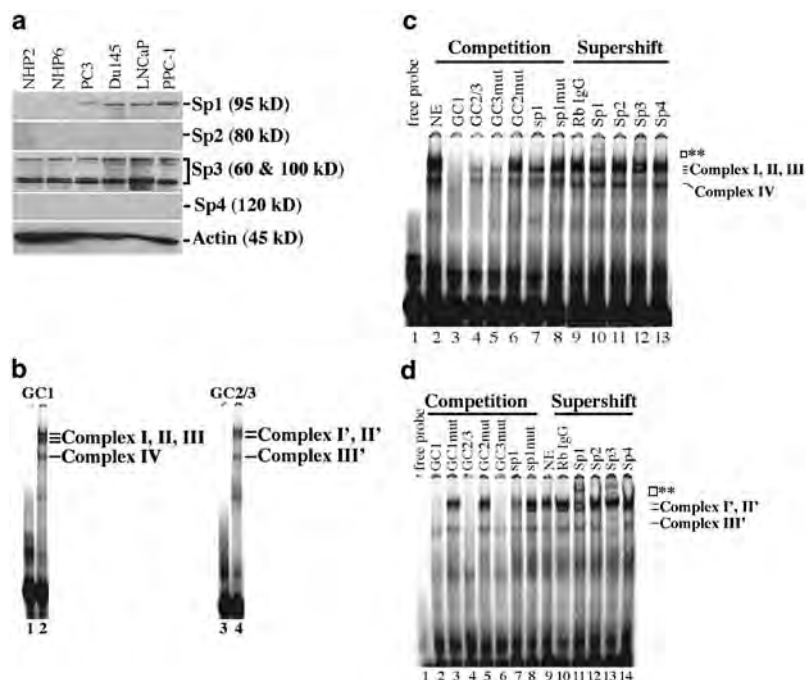
Figure 6 Overexpression of Sp1 and Sp3 proteins in PCa cells and EMSA in PPC1 cells. (**a**) PCa cells express higher levels of Sp1 and Sp3 proteins. Whole-cell lysates (80 μg/lane) from cells indicated were utilized for Western blotting of Sp1–Sp4 proteins. Both NHP2 and NHP6 cells were used at P6. (**b**) EMSA in PPC-1 cells using either GC1 probe (lane 2) or GC2/GC3 probe (lane 4). Lanes 1 and 3 were the NE alone. (**c** and **d**) EMSA competition and supershift assays in PPC-1 cells using either GC1 probe (**c**) or GC2/GC3 (**d**) probe. The legends are similar to those in Figure 5. Data shown in (**c**) and (**d**) were representative of three to four experiments with comparable results. RbIgG, non-immune rabbit IgG

Unlabeled, mutant GC1 probe, in which both GC box 1 and CACCC box were altered (Table 1), slightly reduced the binding of all four complexes, especially complex IV (Figure 5a, lane 4), suggesting that sequence(s) outside of the GC box 1/CACCC region may also help in the formation of these complexes. Unlabeled wild-type GC2/3 probe (Figure 5a, lane 5) as well as consensus Sp1 probe (Figure 5a, lane 8) but not mutant Sp1 probe, also showed strong competing effects, especially on complexes I and II. These results suggest that complexes I and II are formed mostly by transcription factors that bind to GC boxes. Surprisingly, unlabeled GC3 mutant (Figure 5a, lane 6), but not GC2 mutant (Figure 5a, lane 7) probe behaved just like the cold, wild-type GC2/3 probe and demonstrated strong competing effect on complexes I and II. As GC3 mutant retains intact GC box 2, whereas GC2 mutant retains intact GC box 3 (Table 1), these results suggest that the GC box 2 is more important. Supershift experiments revealed that the antibodies to Sp1 and Sp3 formed supershifted bands, whereas antibodies to Sp2 and Sp4 did not (Figure 5a, lanes 10–13). Sp1 appeared to exist in complexes I/II (Figure 5a, lane 10) whereas Sp3 in complex II (Figure 5a, lane 12).

When the GC2/GC3 probe (Table 1) was used, at least three complexes, that is, complex I′–III′, were observed (Figure 5b, lane 2). These complexes were all competed out with unlabeled, wild-type GC1, GC2/GC3, and Sp1 consensus oligonucleotides (Figure 5b, lanes 3, 5, and 8) but not with their corresponding mutants (Figure 5b, lanes 4, 7, and 9). Interestingly, just as observed in Figure 5a (lanes 6 and 7), only the GC3 mutant but not GC2 mutant competed out all three complexes (Figure 5b, lanes 6 and 7). Collectively, these data suggest that, likely, all three complexes are formed on GC box 2. Supershift experiments (Figure 5b, lanes 10–13) revealed that: (1) Sp1 existed in complex I′ whereas Sp3 in complexes II′ and III′ and (2) no supershifted bands were observed with antibodies to Sp2 and Sp4.

Taking advantage of the fact that cancer cells often overexpress Sp family proteins (Black et al., 2001), we also carried out EMSA experiments in PCa cells. As shown in Figure 6a, Sp1 was indeed expressed at much higher levels in PCa cells than in NHP cells. Sp1 was detected as a major ∼95 kDa protein with a minor slower-migrating band, which might represent the phosphorylated Sp1 (Jackson et al., 1990; Black et al., 2001). Sp3, which was detected as a doublet migrating at ∼60 and ∼100 kDa, was detected in all cell types but their levels, especially those of the ∼100 kDa doublet, were also slightly higher in PCa cells (Figure 6a). In contrast, Sp2 and Sp4 could not be detected in any cells (Figure 6a).

As in NHP6 cells (Figure 5), EMSA using GC1 probe detected four distinct complexes, whereas EMSA using GC2/GC3 probed at least three complexes in PPC-1 cells (Figure 6b) as well as in LNCaP, PC3, and Du145 cells (not shown). Competition and supershift experiments in PPC-1 cells using either GC1 probe (Figure 6c)

or GC2/GC3 probe (Figure 6d) revealed banding patterns essentially identical to those observed in NHP6 cells (compare with Figure 5a and b), except that the band intensities in PCa cells were stronger than in NHP6 cells. With GC1 probe, again four complexes were detected (Figure 6c, lane 2), which were all competed out by cold GC1 probe. As in NHP6 cells (Figure 5a), unlabeled wild-type GC2/GC3, GC3 mutant, and Sp1 oligonucleotides competed out primarily complexes I and II (Figure 6c, lanes 4, 5, and 7, respectively). Mutant Sp1 did not have any effect (Figure 6c, lane 8). Unlabeled GC2 mutant failed to show any competition effect (Figure 6c, lane 6). Supershift experiments also revealed banding patterns (Figure 6c) similar to those in NHP6 cells (Figure 5a). EMSA with GC2/GC3 probe revealed three complexes, I′, II′, and III′, which were competed out, to various degrees, by unlabeled wild-type GC1, GC2/3, and Sp1 oligonucleotides but not by their mutants (Figure 6d). As in NHP cells, GC3 mutant but not GC2 mutant showed strong competing effect (Figure 6d, lanes 5 and 6), and Sp1 primarily existed in complex I′ (Figure 6d, lane 10), whereas Sp3 in complexes II′ and III′ (Figure 6d, lane 13). A subtle difference consistently observed between PPC-1 and NHP6 cells was that the complex III′ was not effectively competed out in PPC-1 cells (compare Figure 6d and Figure 5b), perhaps due to higher levels of Sp3 (Figure 6a) and other transcription factors.

Androgen does not directly regulate 15-LOX2 gene expression

15-LOX2 is expressed in differentiated (i.e. luminal) prostate epithelial cells (Shappell *et al.*, 1999; Tang *et al.*, 2002), which are normally regulated by androgen through the AR. We wonder whether 15-LOX2 itself might be directly regulated by androgen. To test this possibility, we first carried out *in silico* analysis, using various tools and databases described earlier, of ~ 16 kb 15-LOX2 DNA sequence upstream of ATG (NM_001141). We did not identify any ARE that matched the consensus sequence GG(T/A)ACANNNTGTTCT (the underlined sequences are the inverted repeat) derived from 29 AREs, except a partially matched ARE with the sequence GGCACAGCTTGTGCC located at -1437 upstream of the TSS (see Figure 8, below). To determine whether this partially matched ARE or some other sequences might regulate 15-LOX2 gene expression, we cloned a ~ 4.0 kb 15-LOX2 promoter fragment from NHP6 cells using primers P2 and P10 (Table 1) that encompasses the partially matched ARE and cloned it into the pGL3-basic vector, that is, $(-3985/+250)$-luc. When transfected into AR-positive LNCaP cells, the $-3985/+250$ fragment showed increased promoter activity over the empty vector, and this increased promoter activity was not affected by androgen, dihydrotestesterone (DHT) (Figure 7a). As expected, the promoter activity of $(-726/+80)$-luc transfected into the LNCaP was not affected by DHT, either (Figure 7a), as there was no

ARE in this region (Figure 1b). Of note, the $-3985/+250$ fragment showed a similar promoter activity to that of $-726/+80$ (Figure 7a), consistent with our earlier findings that the basal promoter activity was mostly located in the GC-rich proximal promoter region. When both promoter constructs were cotransfected with the AR into the AR-negative PPC-1 cells, their promoter activities were similarly not affected by DHT (not shown). In contrast, as a positive control, DHT induced the expression of exogenous 15-LOX2 controlled by androgen-responsive promoter (ARR$_2$PB) (Zhang *et al.*, 2000) in LNCaP cells (Figure 7b, lane 4), which expressed AR but little endogenous 15-LOX2 (Figure 7c, lane 7). Cotransfection of AR further induced the expression of the 15-LOX2 transgene by DHT (Figure 7b, lane 6), although it also induced 15-LOX2 transgene expression in the absence of DHT (Figure 7b, lane 5). Note that the antibody detected a faint nonspecific band that migrated slightly slower than 15-LOX2 (Figure 7b). These results overall are consistent with the lack of authentic ARE in the 15-LOX2 promoter and suggest that: (1) the partially matched ARE does not represent a functional ARE and (2) 15-LOX2 does not appear to be directly regulated by androgen.

To further explore this point, we carried out several sets of experiments in early-passage NHP6 cells (Figure 7c–f). We took advantage of our recent observations that the NHP cells are of the basal cell origin and do not express 15-LOX2 at early passages, that is, passages 2 and 3 (Figure 7c, lane 3). They gradually acquire 15-LOX2 expression by passages 4–6 (Figure 7c, lane 2), and by passage 7 essentially 100% cells express high levels of 15-LOX2 (Bhatia *et al.*, manuscript in preparation). This is the main reason that we performed all luciferase experiments in late passage (i.e. P5–7) NHP6 cells. As shown in Figure 7c (lanes 2 and 3), NHP6 cells at passage 6 expressed mRNAs for both 15-LOX2 and 15-LOX2sva/sv-b, whereas NHP6 cells at passage 3 expressed neither. Late-passage NHP6 cells also showed increased AR mRNA expression (Figure 7c, lanes 2 *vs* 3), although no AR protein could be detected (not shown). When the ARR$_2$PB-15-LOX2 construct was cotransfected with the AR plasmid into the early-passage NHP6 cells, DHT induced strong expression of the mRNA (Figure 7c, lane 6) and protein expression of the 15-LOX2 transgene in AR-positive cells (Figure 7d). A counting of ~ 300 AR-transfected NHP6 (passage 3) cells revealed 100% positivity for 15-LOX2 staining. AR transfection in the absence of DHT did not result in increased 15-LOX2 mRNA or protein expression (not shown). In contrast, transfection of AR into the young NHP6 cells did not result in DHT-inducible expression of endogenous 15-LOX2 (Figure 7c, lane 5; and data not shown). Interestingly, DHT alone slightly upregulated endogenous AR mRNA levels, but the 15-LOX2 mRNA was not induced (Figure 7c, lane 3 *vs* lane 4). Similarly, the low, basal-level promoter activities of $(-726/+80)$-luc or the $(-3985/+250)$-luc transfected into passage 3 NHP6 cells were not affected by the cotransfected AR in the presence or absence of DHT
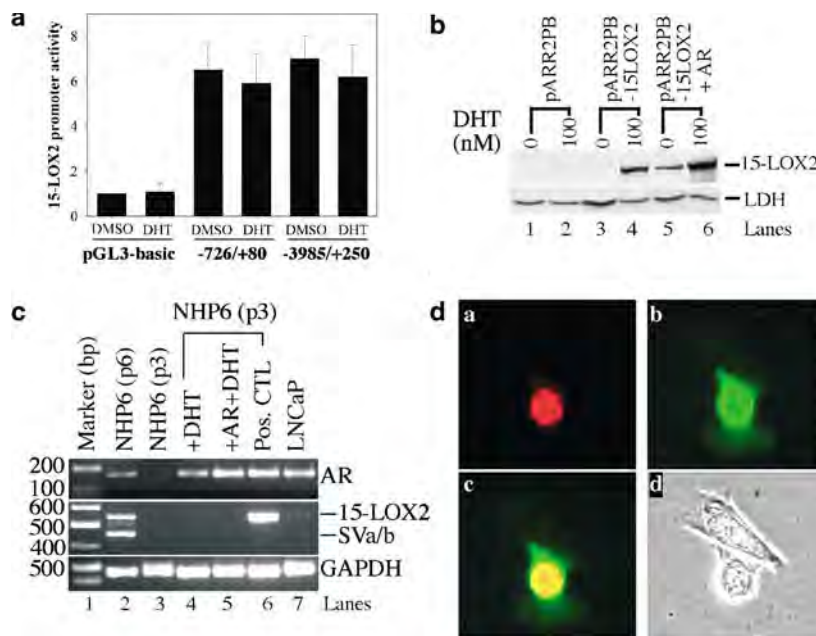
Figure 7 Androgen does not directly regulate 15-LOX2 gene expression. (**a**) Lack of 15-LOX2 promoter activity in LNCaP cells stimulated with DHT. LNCaP cells cultured in charcoal-stripped FBS (48 h) were transfected with the vectors indicated. After 24 h, cells were stimulated with DMSO or DHT (100 nM). Cells were harvested for luciferase activity assays 24 h later and the results were expressed as the relative 15-LOX2 promoter activity. Data represent the mean ± s.e.m. ($n = 5$). (**b**) DHT induces 15-LOX2 transgene expression. LNCaP cells were similarly treated with charcoal-stripped serum as in (**a**), except that cells were transfected with either the empty vector (lanes 1 and 2), pARR$_2$PB-15LOX2 (lanes 3 and 4) or cotransfected with pARR$_2$PB-15LOX2 and AR (lanes 5 and 6). Cells were harvested 24 h after DHT treatment and used for Western blotting of 15-LOX2 (upper panel) or lactate dehydrogenase (LDH; lower panel). (**c**) DHT/AR do not induce endogenous 15-LOX2 mRNA expression. Young (P3) NHP6 cells were either untreated (lane 3) or directly treated with DHT (100 nM) for 72 h (lane 4), or first transfected with AR for 48 h and then stimulated with DHT for 24 h (lane 5), or first transfected with pARR$_2$PB-15LOX2 for 48 h and then stimulated with DHT for 24 h (lane 6, positive control). At the end, cells were harvested and total RNA prepared for RT–PCR of AR (sense: 5′-GAAGCCATTGAGCCAGGTGT-3′; antisense, 5′-TCGTCCACGTGTAAGTT GCG-3′), 15-LOX2, or GAPDH. Late-passage NHP6 cells (lane 2) and LNCaP (lane 7) were used as controls for 15-LOX2 and AR, respectively. (**d**) DHT/AR induce exogenous 15-LOX2 protein expression. NHP6 (P3) cells were transfected with AR and pARR2PB-15-LOX2. After 48 h, cells were stimulated with DHT for 24 h and then dual labeled for AR (**a**) and 15-LOX2 (**b**). Micrograph in (**c**) represents overlays of images a and b and micrograph in (**d**) is the phase-contrast image. The images shown are representative of ∼300 AR-transfected cells analysed, which were all positive for 15-LOX2. Original magnification: ×400
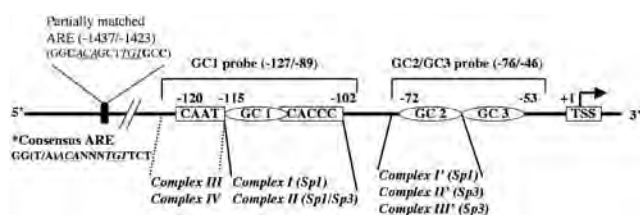

Figure 8 A model depicting the two GC-rich regions and potential transcription factor complexes formed on these regions in the 15-LOX2 gene promoter. Consensus ARE sequence and a partially matched ARE are also shown. TSS, transcription start site

(not shown). These observations in young NHP6 cells thus confirm the earlier conclusion that androgen does not directly or is not sufficient to regulate the 15-LOX2 gene expression.

Discussion

The present study addresses how 15-LOX2 expression may be regulated in NHP cells. The results suggest that:

(1) several Sp1 sites in the proximal promoter region are important *cis* elements regulating the 15-LOX2 promoter activity; (2) Sp1 and Sp3 are two major GC-binding *trans* factors regulating 15-LOX2 gene expression with Sp1 being a positive and Sp3 a negative regulator; (3) multiple complexes form on the GC-rich regions and Sp1 and Sp3 exist in some of these complexes; and (4) androgen does not directly regulate 15-LOX2 gene expression.

Similar to 15-LOX1 and 12-LOX promoters (Kritzik et al., 1997; Kelavkar et al., 1998), the 15-LOX2 promoter is TATAA less, consistent with its constitutive expression in adult NHP cells *in vivo*. Sp1 sites including GC boxes and CACCC boxes (also called GT boxes) have been shown to be responsible for recruiting TATA-binding protein and fixing the TSS on TATAA-less promoters (Strachan and Read, 2000; Black et al., 2001; Levine and Tijan, 2003). *In silico* analysis identifies four potential Sp1 sites in the proximal promoter region of 15-LOX2 gene. Initial evidence that these Sp1 sites may play a role in regulating 15-LOX2 comes from deletion analysis. More definitive evidence comes from site-specific mutagenesis, which suggests that

GC box 2 and/or 3 may be more important than the GC box 1/CACCC box, as well as from EMSA competition experiments, which suggest that GC box 2 is the primary Sp1 site that multiple transcription factors bind.

Interestingly, promoter deletion experiments suggest that the sequence between −726 and −163 might contain a *cis* element(s) that negatively regulate the 15-LOX2 promoter activity. Furthermore, *in silico* analysis has also revealed multiple other perfectly matched transcription factor-binding sites. Many of these *cis* elements, together with their cognate transcription factors, have been implicated in physiological processes such as cell fate determination, proliferation, and differentiation as well as in pathological conditions including tumorigenesis. Conceivably, these elements may participate in the regulation of tissue-specific, differentiation-related, or stage-specific expression of 15-LOX2.

What *trans* factors bind to the Sp1 sites to regulate 15-LOX2 promoter activity? The Sp family proteins, that is, Sp1–Sp4, are the natural candidates as they primarily bind to GC-rich sequences. In the Sp subfamily, Sp1 and Sp3 are ubiquitously expressed and Sp4 is expressed mostly in central nervous and reproductive systems, whereas the expression pattern of Sp2 is largely unknown (Black *et al*., 2001). Sp1, Sp3, and Sp4 share a high affinity for GC boxes bearing consensus sequence GGGGCGGGG, while Sp2 only weakly binds to GT boxes (Kingsley and Winoto, 1992). NHP cells express low levels of Sp1, easily detectable Sp3, and undetectable Sp2 and Sp4 proteins, suggesting that Sp1 and Sp3 may be involved in regulating 15-LOX2 gene expression through binding to GC-rich Sp1 sites. Indeed, using SL2 cells that lack all endogenous Sp proteins, we demonstrate that Sp1 *can* activate 15-LOX2 promoter. Three additional lines of evidence establish that the Sp1 protein *is required* for 15-LOX2 gene expression. First, DN-Sp1 inhibits the 15-LOX2 promoter activity in transfected NHP cells. Second, the Sp1 inhibitor, MMA also inhibits 15-LOX2 promoter activity. Third, most importantly, MMA, in a time- and dose-dependent manner, suppresses endogenous 15-LOX2 mRNA expression.

In the Sp subfamily, Sp3 is the only protein that can either positively or negatively modulate the Sp1-dependent gene expression. Our subsequent experiments demonstrate that Sp3 dose dependently inhibits the Sp1-activated 15-LOX2 promoter activity in SL2 cells. Furthermore, Sp3 inhibits the 15-LOX2 promoter activity as well as endogenous 15-LOX2 expression in transfected NHP cells. Together, these results establish that Sp1 and Sp3 are biologically relevant and essential regulators of the 15-LOX2 gene expression with Sp1 being an activator and Sp3 an inhibitor via antagonizing Sp1 activity.

EMSA experiments demonstrate that multiple complexes form at the two GC-rich regions and both Sp1 and Sp3 exist in some of these complexes (Figure 8). Complex I and II are competed out by consensus Sp1 and GC2/GC3 oligonucleotides, suggesting that these two complexes are mainly formed of GC-binding proteins. Indeed, supershift experiments reveal the presence of Sp1 in complexes I and II and Sp3 mainly in complex II. What is the molecular nature of complexes III and IV that are competed out by unlabeled GC1 oligonucleotides but not significantly by Sp1 and GC2/GC3 oligonucleotides? Likely candidates may include those transcription factors (e.g. NF-1, NF-Y, and C/EBP) that normally bind to the CAAT box (Strachan and Read, 2000). In fact, NF-Y and Sp1 have recently been shown to cooperate in regulating the expression of several genes (Yamada *et al*., 2000; Liang *et al*., 2001).

When EMSA experiments are carried out using the GC2/GC3 probe, three major complexes are observed, which can be competed out, to different degrees, by GC1, GC2/3, or Sp1 oligonucleotides. Supershift experiments reveal that Sp1 exists in complex I′ and Sp3 in complexes II′ and III′ (Figure 8). Much to our surprise, the unlabeled GC3 mutant probe, but not mutant GC2 probe, behaves exactly like the wild-type GC2/3 oligonucleotide in competing out these complexes. The contrasting effects of GC2 and GC3 mutants are also observed in competition experiments using GC1 probe. These observations strongly suggest that the GC box 2 is *the* major binding site for Sp1, Sp3, and perhaps other related proteins (Figure 8), consistent with the site-specific mutagenesis data.

That 15-LOX2 is expressed exclusively in the androgen-sensitive luminal cells *in vivo* promoted us to address whether 15-LOX2 might be directly regulated by androgen. Several pieces of evidence argue against this possibility. First, there is no authentic ARE in the 16 kb upstream sequence of 15-LOX2 promoter. Second, although there is a partially matched ARE at ∼−1.4 kb kb location (Figure 8), a 4 kb fragment containing this partially matched ARE does not possess DHT-inducible promoter activity. This result is not surprising as this partially matched ARE has three conspicuous base differences (bold in Figure 8) from the ARE consensus. Third, androgen and AR do not induce endogenous 15-LOX2 gene expression in young NHP cells, although they can readily activate a 15-LOX2 transgene driven by ARR_2PB promoter. Fourth, we have observed that 15-LOX2 expression is cell autonomously upregulated in NHP cells cultured in the absence of androgen or serum, and that 15-LOX2 gene expression in human prostates comes after PSA expression, probably as a consequence of prostate differentiation, and appears to be involved in replicative cell senescence (Bhatia *et al*., manuscript in preparation). Finally, that androgen does not directly regulate 15-LOX2 gene expression is also consistent with clinical data that PCa tend to have heightened AR activity leading to increased PSA production, but they downregulate or lose 15-LOX2 expression. It should be noted that, although our data do not indicate a direct regulation of 15-LOX2 by androgen, it is still possible that androgen may indirectly regulate 15-LOX2 expression or its function.

Materials and methods

Cells and reagents

NHP1–NHP6 cells were cultured in serum- and androgen-free, PrEBM medium (Clonetics) supplemented with insulin, EGF, hydrocortisone, bovine pituitary extract, and cholera toxin, and used at passages 3–7 (Chopra et al., 1996; Tang et al., 1998, 2002; Bhatia et al., 2003). PCa cell lines, PPC-1, PC3, LNCaP, and Du145, were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics.

Luciferase reporter plasmid, pGL3-basic, was purchased from Promega. Rabbit polyclonal anti-15-LOX2 antibody was described previously (Tang et al., 2002). Rabbit polyclonal antibodies to Sp1–Sp4 were obtained from Santa Cruz. Liposome FuGene 6 was bought from Roche. All chemicals were bought from Sigma unless specified otherwise.

Cloning of immediate upstream 15-LOX2 promoter region and mapping of transcription start site (TSS)

The ~1.2 kb fragment upstream of ATG was cloned using primers P3 and P8 (Table 1) and genomic DNA prepared from several different NHP cell strains. The primer sequences were based on the genomic sequences deposited in the GenBank (Accession numbers AJ305028–AJ305031). A primer extension analysis was performed to determine the TSS of the 15-LOX2 gene using standard protocol (Sambrook and Russell, 2001). Briefly, the reverse primer P8 (Table 1) was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The labeled primer was then annealed (65°C × 90 min) to 0.5 µg mRNA purified from NHP2 cells and extended (42°C × 60 min) with Super-Script II reverse transcriptase (Gibco). In vitro transcribed cDNA fragments were electrophoresed on denaturing polyacrylamide gels containing 8 M urea in parallel to a sequencing reaction (using the 1.2 kb fragment as the template) as a size marker.

Generation of the 15-LOX2 promoter deletion mutants and analysis of promoter activity

The 1.2 kb 15-LOX2 promoter (i.e. −1116/+80) fragment was used as the template to generate a series of truncated deletion mutants using PCR primers indicated in Table 1. An XhoI site (CTCGAG) was incorporated into the 5′-ends of the PCR primers and the resultant PCR fragments were first cloned into the pCRII-TOPO cloning vector and subsequently cloned into pGL3-basic. The orientation and sequence of each insert were confirmed by restriction digestion and sequencing. For luciferase reporter assays, NHP or PCa cells grown in six-well culture plates (4–5 × 10^4 cells/well) were transiently transfected, in triplicate, with either empty vector (i.e. pGL3-basic) or various luc constructs (2 µg plasmid/well) together with the β-gal plasmid (0.125 µg plasmid/well) to normalize the transfection efficiency. One set of the triplicate wells was mock-transfected. Cells were harvested 48 h after transfection by scraping into the 1 × lysis buffer (Promega). Lysates containing equal amounts of protein were assayed for luciferase activity using luciferase assay kit (Promega). β-Galactosidase activity was measured using Tropix Galacto-Light Plus assay system. After subtracting the baseline values, the relative luciferase unit (RLU) activity was determined as the ratio of luciferase/β-gal activities. The relative promoter activities were expressed as either RLU or relative values (i.e. % or fold increase).

Site-specific mutagenesis of Sp1 sites

Site-specific mutagenesis was performed with the Quick-Change Site-Specific Mutagenesis system (Stratagene) using the (−163/+80)-luc as template with the PCR primers and individual mutations indicated in Table 1. Double mutations of GC box 1 and GC box 2 or GC box 1 and GC box 3 were made using the (−163/+80)-luc containing mutated GC box 1 as the template to further mutate GC box 2 or 3. The successfully mutated sequences were confirmed by restriction digestion and sequencing.

Involvement of Sp1 family proteins in regulating 15-LOX2 gene expression D. melanogaster

Schneider SL2 cells were cotransfected with (−726/+80)-luc or (−102/+80)-luc and an Sp1 or Sp3 expression vector driven by Drosophila actin promoter (i.e. pPacSp1 or pPacSp3; Kumar and Butler, 1997). The Sp1 plasmid encodes amino acids 83–758 of human Sp1 under the control of the Drosophila actin 5C promoter. In another experiment, NHP6 cells were cotransfected with several luc constructs together with a dominant-negative (DN) Sp1 expression vector (pEBG-Sp1; Ptersohn and Thiel, 1996; Grinstein et al., 2002) or a mammalian Sp3 expression vector (Kumar and Butler, 1997). Alternatively, NHP6 cells were treated with an Sp1-specific chemical inhibitor mithramycin A (MMA; Kaluz et al., 2003) at 200 nM at the time of transfection of promoter constructs. All transfection experiments were carried out as described above. Finally, NHP6 cells were transfected with DN-Sp1 or Sp3 for various time periods or treated with various doses of MMA for different time periods followed by RNA isolation and RT–PCR analysis using 15-LOX2-specific primers C-D (Tang et al., 2002; Bhatia et al., 2003).

EMSA

Nuclear extract (NE) was prepared from NHP2, NHP6, or various PCa cells (Bhatia et al., 2003). For EMSA, three different double-stranded oligonucleotide probes (Table 1) were used. A probe containing consensus Sp1 site and the corresponding mutant probe were purchased from Santa Cruz. The second probe (i.e. GC1 probe) contained wild-type or mutant overlapping GC box 1 and CACCC box. The third probe (i.e. GC2/GC3 or GC2/3 probe) was derived from the proximal promoter of 15-LOX2 that contained GC box 2 and 3 (Table 1). Two mutant probes, that is, GC2 or GC3 mutant, were designed for the GC2/GC3 probe (Table 1). In all, 10 pmol of DNA fragment were end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. For EMSA, 6 µg NE was incubated in a 20 µl reaction containing 100 000 c.p.m. probe, 20 mM HEPES (pH 7.9), 5% glycerol, 2 mM MgCl$_2$, 0.2 mM EDTA, 50 mM KCl, and 0.5 mg/ml BSA. After 30 min incubation at room temperature, the DNA–protein complexes were separated on 5% polyacrylamide gel containing 5% glycerol and 0.5 × TBE. Competition assays used 100 × cold unlabeled probes. Supershift assays were performed using the antibodies (1 µg) to Sp1–Sp4.

Western blotting

Western blotting was performed using whole-cell lysates as described previously (Tang et al., 2002).

Potential effect of androgen on 15-LOX2 gene expression

Several sets of experiments including in silico promoter analysis, cloning, and characterization of a ~4.0 kb. 15-LOX2 promoter sequence, luciferase transfection, RT–PCR, and immunostaining of 15-LOX2 upon transfection of exogenous AR were carried out to examine the potential role

of androgen in 15-LOX2 gene expression (detailed in Results). As a positive control, we cloned 15-LOX2 cDNA under the control of an ARR$_2$PB (Zhang *et al.*, 2000). The vector, pARR$_2$PB-15LOX2, or various 15-LOX2 promoter constructs, was transfected into AR-positive LNCaP cells, which had been cultured in charcoal-stripped serum for 48 h. At 48 h after transfection, DHT was added to the culture medium at a final concentration of 100 nM. Cells were harvested, 24 h after DHT stimulation, for either luciferase activity measurement or Western blotting for 15-LOX2. In other experiments, these vectors were transfected into AR-negative cells by cotransfecting with a human AR expression plasmid and then RT–PCR (for 15-LOX2 expression), immmunofluorescence staining (for both 15-LOX2 and AR), or luciferase assays (for 15-LOX2 promoter activity) were carried out.

Abbreviations

AA, arachidonic acid; AR, androgen receptor; DN, dominant negative; EMSA, electrophoretic mobility shift assay; LOX, lipoxygenase; 15-LOX2, 15-lipoxygenase 2; 15-LOX2sv-a/b/c, 15-LOX2 splice variant a, b or c; MMA, mithramycin A; NE, nuclear extract; NHP, normal human prostate epithelial cells; PCa, prostate cancer; RLU, relative luciferase unit; TSS, transcription start site.

References

Bhatia B, Maldonado CJ, Tang S, Chandra D, Klein RD, Chopra D, Shappell SB, Yang P, Newman RA and Tang DG. (2003). *J. Biol. Chem.*, **278**, 25091–25100.

Black AR, Black JD and Azizkhan-Clifford J. (2001). *J. Cell. Physiol.*, **188**, 143–160.

Blais A, Monte D, Pouiot F and Labrie C. (2002). *J. Biol. Chem.*, **277**, 31679–31693.

Brash AR, Boeglin WE and Chang MS. (1997). *Proc. Natl. Acad. Sci. USA*, **94**, 6148–6152.

Chen BK and Chang WC. (2000). *Proc. Natl. Acad. USA*, **97**, 10406–10411.

Chopra DP, Grignon DJ, Joiakim A, Mathieu PA, Mohamed A, Sakr WA, Powell IJ and Sarkar FH. (1996). *J. Cell. Physiol.*, **169**, 269–280.

Frith MC, Hansen U and Weng Z. (2001). *Bioinformatics*, **17**, 878–889.

Garcia-Ruiz I, de la Torre P, Diaz T, Esteban E, Fernandez I, Munoz-Yague T and Solis-Herruzo JA. (2002). *J. Biol. Chem.*, **277**, 30551–30558.

Grinstein E, Jundt F, Weinert I, Wernet P and Yoyer H-D. (2002). *Oncogene*, **21**, 1485–1492.

Hagen G, Muller S, Beato M and Suske G. (1994). *EMBO J.*, **13**, 3843–3851.

Heinemeyer T, Wingender E, Reuter I, Hermjakob H, Kel A, Kel O, Ignatieva E, Ananko E, Podkolodnaya O, Kolpakov F, Podkolodny N and Kolchanov N. (1998). *Nucleic Acid Res.*, **26**, 364–370.

Hong C, Kim H-A, Firestone GL and Bjeldanes LF. (2002). *Carcinogenesis*, **23**, 1297–1305.

Huang L, Sowa Y, Sakai T and Pardee AB. (2000). *Oncogene*, **19**, 5712–5719.

Jackson SP, MacDonald JJ, Lees-Miller S and Tijan R. (1990). *Cell*, **63**, 155–165.

Kaluz S, Kaluzova M and Stanbridge EJ. (2003). *Cancer Res.*, **63**, 917–922.

Kelavkar U, Wang S, Montero A, Murtagh J, Shah K and Badr K. (1998). *Mol. Biol. Reports*, **25**, 173–182.

Kilty I, Alison L and Vickers PJ. (1999). *Eur. J. Biochem.*, **266**, 83–93.

Kingsley C and Winoto A. (1992). *Mol. Cell. Biol.*, **12**, 4251–4261.

Kritzik MR, Ziober AF, Dicharry S, Conrad DJ and Sigal E. (1997). *Biochim. Biophys. Acta*, **1352**, 267–281.

Kumar AP and Butler AP. (1997). *Nucleic Acid Res.*, **25**, 2012–2019.

Levine M and Tijan R. (2003). *Nature*, **424**, 147–151.

Liang F, Schaufele F and Gardner DG. (2001). *J. Biol. Chem.*, **276**, 1516–1522.

Locker J. (1993). *Gene Transcription: A Practical Approach* Hames BD and Higgins SJ (eds). Oxford University Press: Oxford, UK, pp. 321–345.

Matys V, Fricke E, Gefferes R, Goβling E, Haubrock M, Hehl R, Hornischer K, Karas D, Kel AE, Kel-Margoulis OV, Kloos D-U, Land S, Lewicki-Potapov B, Michael H, Munch R, Reuter I, Rotert S, Saxel H, Scheer M, Thiele S and Wingender E. (2000). *Nucleic Acid Res.*, **28**, 316–319.

Ptersohn D and Thiel G. (1996). *Eur. J. Biochem.*, **239**, 827–834.

Sambrook J and Russell DW. (2001). *Molecular Cloning: A Laboratory Manual* 3rd edn., Vol. 1. CSHL Press, Cold Spring Harbor Laboratory: New York, pp. 7.75–7.81.

Schafer G, Cramer T, Suske G, Kemner W, Wiedenmann B and Hocker M. (2003). *J. Biol. Chem.*, **278**, 8190–8198.

Shappell SB, Boeglin WE, Olson SJ, Kasper S and Brash AR. (1999). *Am. J. Pathol.*, **155**, 235–245.

Strachan T and Read AP. (2000). *Human Molecular Genetics* 2nd edn. Wiley-Liss: New York, pp. 169–208.

Tang DG, Li L, Chopra D and Porter AT. (1998). *Cancer Res.*, **58**, 3466–3479.

Tang S, Bhatia B, Maldonado C, Yang P, Newman RA, Liu J, Chandra D, Traag J, Klein RD, Fischer SM, Chopra D, Shen J, Zhau H, Chung LWK and Tang DG. (2002). *J. Biol. Chem.*, **277**, 16189–16201.

Won J, Yim J and Kim TK. (2002). *J. Biol. Chem.*, **277**, 38230–38238.

Yamada K, Tanaka T, Miyamoto K and Noguchi T. (2000). *J. Biol. Chem.*, **275**, 18129–18137.

Zhang J, Thomas TZ, Kasper S and Matusik RJ. (2000). *Endocrinology*, **141**, 4698–4710.

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Cell-autonomous induction of functional tumor suppressor 15-lipoxygenase 2 (15-LOX2) contributes to replicative senescence of human prostate progenitor cells

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Normal human prostatic (NHP) epithelial cells undergo senescence *in vitro* and *in vivo*, but little is known about the tissue-specific molecular mechanisms. Here we first characterize young primary NHP cells as CK5^{+}/CK18^{+} intermediate basal cells that also express several other putative stem/progenitor cell markers including p63, CD44, $\alpha 2\beta 1$, and hTERT. When cultured in serum- and androgen-free medium, NHP cells gradually lose the expression of these markers, slow down in proliferation, and enter senescence. Several pieces of evidence implicate 15-lipoxygenase 2 (15-LOX2), a molecule with a restricted tissue expression and most abundantly expressed in adult human prostate, in the replicative senescence of NHP cells. *First*, the 15-LOX2 promoter activity and the mRNA and protein levels of 15-LOX2 and its multiple splice variants are upregulated in serially passaged NHP cells, which precede replicative senescence and occur in a cell-autonomous manner. *Second*, all immortalized prostate epithelial cells and prostate cancer cells do not express 15-LOX2. *Third*, PCa cells stably transfected with 15-LOX2 or 15-LOX2sv-b, a splice variant that does not possess arachidonate-metabolizing activity, show a passage-related senescence-like phenotype. *Fourth*, infection of early-passage NHP cells with retroviral vectors encoding 15-LOX2 or 15-LOX2sv-b induces partial cell-cycle arrest and big and flat senescence-like phenotype. *Finally*, 15-LOX2 protein expression in human prostate correlates with age. Together, these data suggest that 15-LOX2 may represent an endogenous prostate senescence gene and its tumor-suppressing functions might be associated with its ability to induce cell senescence.

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Introduction

Human prostatic glands consist of two major epithelial cell types: basal and secretory (lumenal). Basal cells express cytokeratin (CK) 5 and 14, whereas lumenal cells, which represent differentiated cells, express CK8 and 18, androgen receptor (AR), prostate-specific antigen (PSA) prostatic acid phosphatase (PAP), CD57 (Liu *et al.*, 1997), and 15-LOX2 (Shappell *et al.*, 1999; Tang *et al.*, 2002). Evidence exists that in human prostate the basal cell compartment may contain putative stem and progenitor cells. *First*, ~80% of the proliferating cells are localized in the basal layer (Bonkhoff *et al.*, 1994). *Second*, the majority of proliferating cells in the early outgrowth of the prostate explants are of the basal cell nature (Robinson *et al.*, 1998; Hudson *et al.*, 2000; Tran *et al.*, 2002; Garraway *et al.*, 2003). *Third*, some basal cells seem to have the ability to differentiate into lumenal cells (Robinson *et al.*, 1998). *Finally*, several molecules known to play an important role in maintaining the stem/progenitor cell self-renewal and differentiation, including Notch-1 (Shou *et al.*, 2001) and p63 (Signoretti *et al.*, 2000), localize exclusively in the basal cell compartment in human prostate.

Recently, multiple adult human organs have been shown to contain stem cells (SC), that is, adult SC (Raff, 2003). Adult human prostate SC, which have not been definitively identified, are thought to localize in the basal cell compartment (Kinbara *et al.*, 1996; Hudson *et al.*, 2000) and appear to preferentially express cell surface molecules CD44 (Liu *et al.*, 1997), $\alpha 2\beta 1$ (Collins *et al.*, 2001), and CD133 (Richardson *et al.*, 2004). The existence of SC in adult human prostate is supported by the ability of a small population of cells to form glandular-like structures in reconstituted systems (Hudson *et al.*, 2000; Collins *et al.*, 2001; Richardson *et al.*, 2004). A small population of CK5 and CK18 double-positive (CK5^{+}/CK18^{+}) cells, called intermediate basal cells, has also been proposed to be prostate stem/progenitor cells (van Leenders *et al.*, 2000; Schalken and van Leenders, 2003).

Adult human prostate is susceptible to two proliferative diseases: benign prostate hyperplasia (BPH), in which stromal cells are the major expanded cells, and prostate cancer (PCa), in which deregulated proliferation occurs mainly in the epithelial compartment. Among a multitude of environmental and genetic factors favoring PCa development, aging is the most significant risk factor: 15–30% of males > 50 years and as many as 80% of the males > 80 years harbor foci of PCa (Ruijter et al., 1999). How aging contributes to PCa development remains an enigma. Cultured NHP cells undergo replicative senescence after a period of proliferation and the process seems to involve the activation of both p16/pRb and p53/p21 pathways (Jarrard et al., 1999; Sandhu et al., 2000; Schwarze et al., 2001; Untergasser et al., 2002). Since replicative senescence is considered a barrier to immortalization and transformation (Hanahan and Weinberg, 2000; Wright and Shay, 2001; Schmitt et al., 2002), it is not surprising that multiple molecules (e.g., Rb, p53, and p16) involved in regulating cell senescence have been implicated in PCa development. SA-βgal-positive, senescent NHP cells have been detected in enlarged BPH prostates (Choi et al., 2000; Castro et al., 2003), but the roles of these cells in the etiology of BPH or PCa remain unclear.

Although molecules commonly involved in regulating replicative cell senescence have been implicated in NHP cell senescence, prostate-specific molecules that may play a specific role in NHP cell senescence have not been reported. Here we present evidence that 15-LOX2, which is most abundantly expressed in adult human prostate, is involved in NHP cell senescence.

Results

Characterization of NHP cells as CK5⁺/CK18⁺ intermediate basal cells that also express several other stem/progenitor cell markers

We first characterized primary NHP cells derived from multiple donors including NHP2 from a 59-year-old donor, NHP4 from a 17-year-old donor, NHP6 from a 28-year-old donor, and NHP7 from a 14-year-old donor. We generally obtained these cells at passage 2 (P2). Immunofluorescent staining revealed that all these NHP cells at P2 were CK5⁺/CK18⁺ and also expressed several other putative stem/progenitor cell markers including p63, hTERT, $\alpha 2\beta 1$, and CD44 (not shown). None of the NHP cells at P2 expressed lumenal markers 15-LOX2, AR, PSA, PAP, or CD57 (not shown).

NHP cells lose the progenitor cell markers in culture

Next, we followed changes in NHP cells, which had been constantly cultured and subcultured in serum- and androgen-free medium containing one survival factor (i.e., insulin) and one mitogen (i.e., EGF). NHP7 cells at P2 had undergone 19 population doublings (PDs) and 37% of the cells were proliferating upon a 4 h-BrdU pulse (Figure 1A, top). All cells were CK5⁺/CK18⁺

(Figure 1Aa–c) and positive for $\alpha 2\beta 1$ (Figure 1Ba), CD44 (Figure 1Be), and p63 (Figure 1Bi). At P3, NHP7 cells had undergone 22 PDs and proliferating cells dropped to 13% (Figure 1A). Accompanying the slowing down in cell proliferation were significantly increased cell sizes (Figures 1Ad–f and 2Bb, f). Most cells were still CK5⁺/CK18⁺ but some cells showed reduced CK18 expression (Figure 1Ad–f). Cell surface expression of $\alpha 2\beta 1$ (Figure 1Bb) and CD44 (Figure 1Bf) was observed in most cells, although more prominent cell–cell border staining was noticed for both molecules. Most cells still showed nuclear staining of p63 (Figure 1Bj).

By P4, NHP7 cells underwent only one extra PD and ~9% of the cells incorporated BrdU (Figure 1A). One of the most prominent changes was the increased numbers of cells that had reduced or lost CK18 expression (Figure 1Ah), leading to significantly reduced numbers of CK5⁺/CK18⁺ cells (Figure 1Ai). The expression levels of $\alpha 2\beta 1$ (Figure 1Bc), CD44 (Figure 1Bg), and p63 (Figure 1Ak) were also significantly decreased in the majority of the cells. Some cells even completely lost the expression of these markers (e.g., Figure 1Bg, arrow). By P5, there was no further increase in PD and no cells incorporated BrdU (Figure 1A) upon a 4 h-pulse. All NHP7 cells at P5 had lost p63 expression (Figure 1Bl) and most (~95%) had lost $\alpha 2\beta 1$ (Figure 1Bd) and CD44 (Figure 1Bh) expression. Most cells also lost CK18 expression (e.g., Figure 1Aj–l) and the numbers of CK5⁺/CK18⁺ cells decreased to ~5%.

To determine whether serially cultured NHP7 cells acquired any characteristics associated with differentiation, we stained cells of various passages for lumenal markers including CD57, PSA, AR, and PAP. We did not observe any positive cells for all four molecules (not shown). These observations together suggest that, as NHP7 cells gradually slow down in cell-cycle progression and approach their proliferative lifespan, they lose the expression of stem/progenitor cell markers without gaining differentiation markers. Similar results were also observed in serially passaged NHP6 cells (not shown).

Cell-autonomous upregulation of 15-LOX2 accompanies NHP cell senescence

The cell-cycle slowdown, loss of stem/progenitor properties, and prominent increases in cell sizes together suggest that the serially cultured NHP cells may be entering replicative senescence. We therefore examined this possibility, first, in NHP6 cells. NHP6 cells also showed an incremental decrease in their proliferative capacity as revealed by cumulative PDs and BrdU labeling (Figure 2A and C). As NHP6 cells declined in proliferation, many of the cells at P5 displayed enlarged and flattened morphology, contained prominent intracellular vacuoles, and stained positive for SA-βgal (Figure 2A and C), a marker of senescence (Dimri et al., 1995), suggesting that these cells were becoming senescent. By comparison, no NHP6 cells at ⩽P4 were stained positive for SA-βgal (Figure 2A and C). At P6 and P7, SA-βgal⁺ NHP6 cells increased to ~50% and 90%, respectively (Figure 2A and C). On these
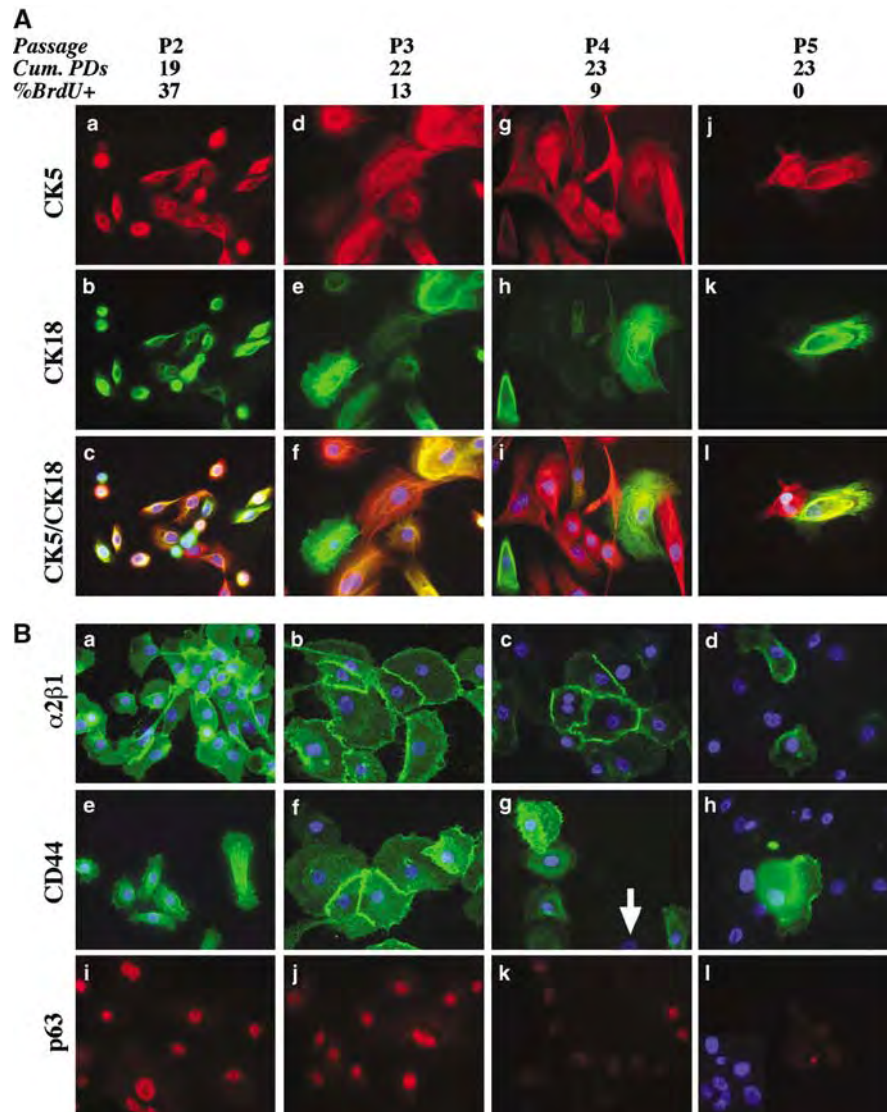
Figure 1 Cultured NHP cells gradually lose stem/progenitor cell properties. (**A**) NHP7 cells were double-stained for CK5 and CK18 and nuclei were counterstained by DAPI. The passage number, cumulative (cum.) PDs, and proliferating (i.e., BrdU+) cells are indicated on top. (**B**) NHP6 cells were stained for α2β1 (**a–d**), CD44 (**e–h**), or p63 (**i, j**). The arrow in (**g**) indicates a cell that has lost CD44 expression. The inset in (**l**) shows the nuclei of the cells stained for p63. Original magnifications: × 400

observations, we designated the NHP6 cells at P2–P4 as young, P5–P6 as presenescent, and P7–P8 as senescent (Figure 2C).

Previously, we observed that primary NHP cells that expressed 15-LOX2, a molecule with a limited tissue distribution (i.e., prostate, lung, hair root, and cornea) and most abundantly expressed in adult prostate (Brash *et al.*, 1997; Kilty *et al.*, 1999), were generally big, flat, and cell-cycle arrested (Tang *et al.*, 2002), raising the possibility that the 15-LOX2 may be associated with the NHP cell senescence. To test this possibility, we carried out triple staining for 15-LOX2, SA-βgal, and BrdU. The results indeed revealed a significantly increased 15-LOX2 expression in NHP6 cells as a function of cell-cycle arrest and replicative senescence (Figure 2A–C). At P2, no NHP6 cells stained positive for 15-LOX2 (not shown). At P3, ~30% NHP6 cells were positive for 15-

LOX2 (Figure 2A) and most of the 15-LOX2+-NHP6 cells were BrdU-negative (Figure 2Cc). In contrast, ~35% of the P3 15-LOX2-negative NHP6 cells were BrdU-positive (Figure 2Cc; Tang *et al.*, 2002). By P5, ~70% NHP6 cells became 15-LOX2+ and ~30% of the cells were SA-βgal+ (Figure 2A and C). A 4-h BrdU pulse no longer labeled any proliferating cells (Figure 2Cf). By P7, nearly all NHP6 cells became strongly 15-LOX2+ and ~90% NHP6 cells stained positive for SA-βgal+ (Figure 2A and C). There was a good correlation between SA-βgal staining and 15-LOX2 expression, that is, cells that were strongly 15-LOX2+ were also strongly SA-βgal+ (Figure 2Ce, f and Ch, i). The increased 15-LOX2 expression in cultured NHP6 cells was verified by Western blotting (see Figure 4A). Interestingly, at all passages analysed, the percentage of 15-LOX2+ cells was higher than that of the SA-βgal+ cells (Figure 2A)
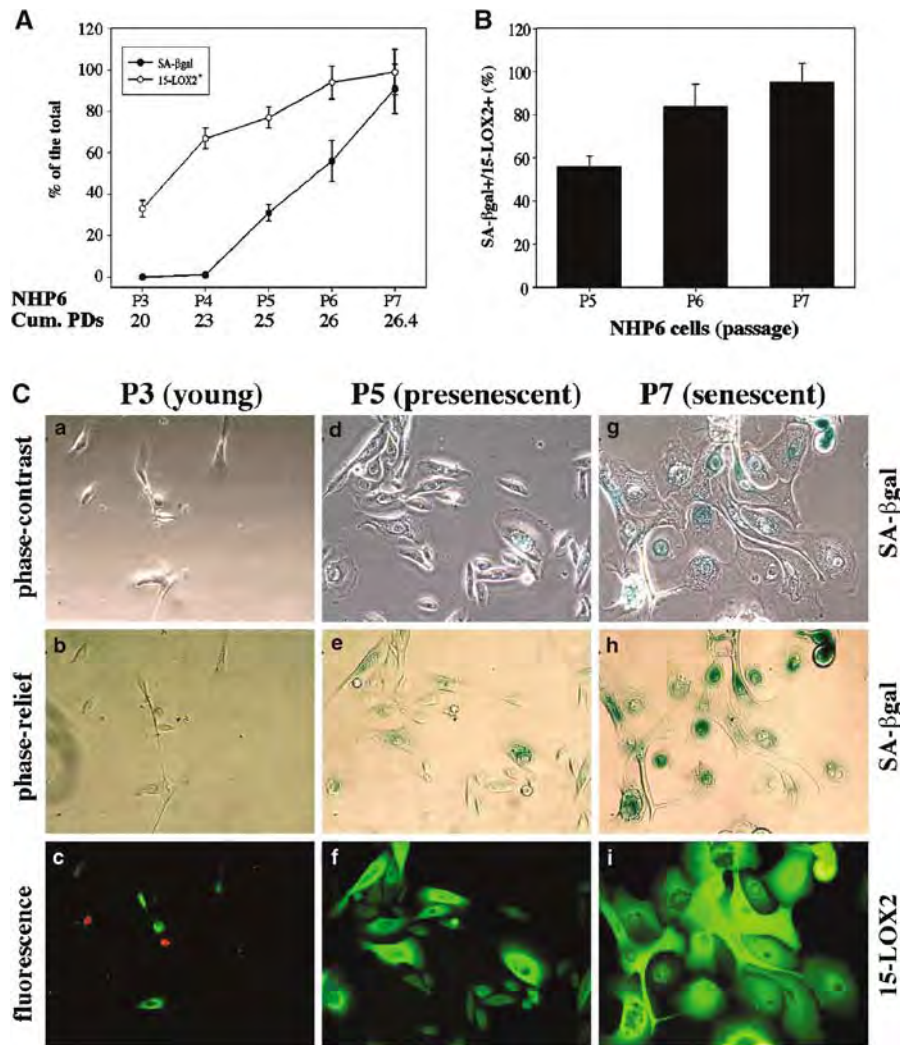
Figure 2 Induction of 15-LOX2 expression in NHP6 cells accompanies replicative cell senescence. (**A**) Quantifications of SA-βgal +
and 15-LOX2 + cells. Triplicate flasks were used in staining and an average of 600–1200 cells was scored for each passage. The bars
represent the mean $\pm$ s.e.m. derived from two separate experiments. Indicated at the bottom are passage numbers and the
corresponding PDs. (**B**) Quantification of SA-βgal and 15-LOX2 double-positive cells in NHP6 cultures. (**C**) Representative
microphotographs showing NHP6 cells at P3, P5, and P7 stained for SA-βgal (top and middle panels), BrdU (red), and 15-LOX2
(green). The images in the middle panels were taken using the phase-relief contrast filters in order to clearly show the SA-βgal staining.
For BrdU staining, cells were pulsed for 4 h. Original magnifications, $\times$ 200

and the 15-LOX2$^+$ cells were much bigger than the 15-
LOX2$^-$ cells (Figure 2C). A differential counting of the
% of SA-βgal$^+$ cells in the 15-LOX2$^+$ population
revealed that $\sim$ 55 and 90% of the 15-LOX2$^+$-cells
were positive for SA-βgal at P3 and P7, respectively
(Figure 2B). Of note, cell nuclei generally accumu-
lated less 15-LOX2 such that many 15-LOX2$^+$
cells appeared to have a hole in the nuclear area
(Figure 2Cc, f, i).

Similar 15-LOX2 upregulation was also observed in
serially passaged NHP2 and NHP7 cells (Figure 3).
NHP2 cells underwent $\sim$ 29 PDs by P5–P6 (Figure 3A)
and 15-LOX2 expression in NHP2 cells was also
inversely correlated with cell proliferation (Figure 3B).
At P3, $\sim$ 35% of the NHP2 cells were actively
incorporating BrdU (Figure 3B) and most of the BrdU$^+$

cells were negative for 15-LOX2 (Figure 3Dc). By
contrast, $\sim$ 10% of the cells were 15-LOX2-positive
(Figure 3B), most of which did not incorporate BrdU
(Figure 3Dc). SA-βgal$^+$ cells were < 2% (Figure 3Db).
By P4–P5, 50–60% of NHP2 cells became 15-LOX2$^+$
and $\sim$ 20% of the cells were SA-βgal$^+$ (Figure 3B and
Dd–f). A 4 h-BrdU pulse no longer labeled any cells
(Figure 3Df). By P6, $\sim$ 100% of NHP2 cells became 15-
LOX2$^+$ and 80% SA-βgal$^+$ (Figure 3Dg–i). Again there
was a good correlation between SA-βgal staining
intensity and 15-LOX2 level (Figures 3De, f and 4Dh,
i) and 15-LOX2$^+$ cells were much bigger than 15-
LOX2$^-$ cells (Figure 3Dc, f, i). Also, senescent NHP2
cells showed prominent intracellular vacuoles and
appeared to accumulate less 15-LOX2 in the nuclei in
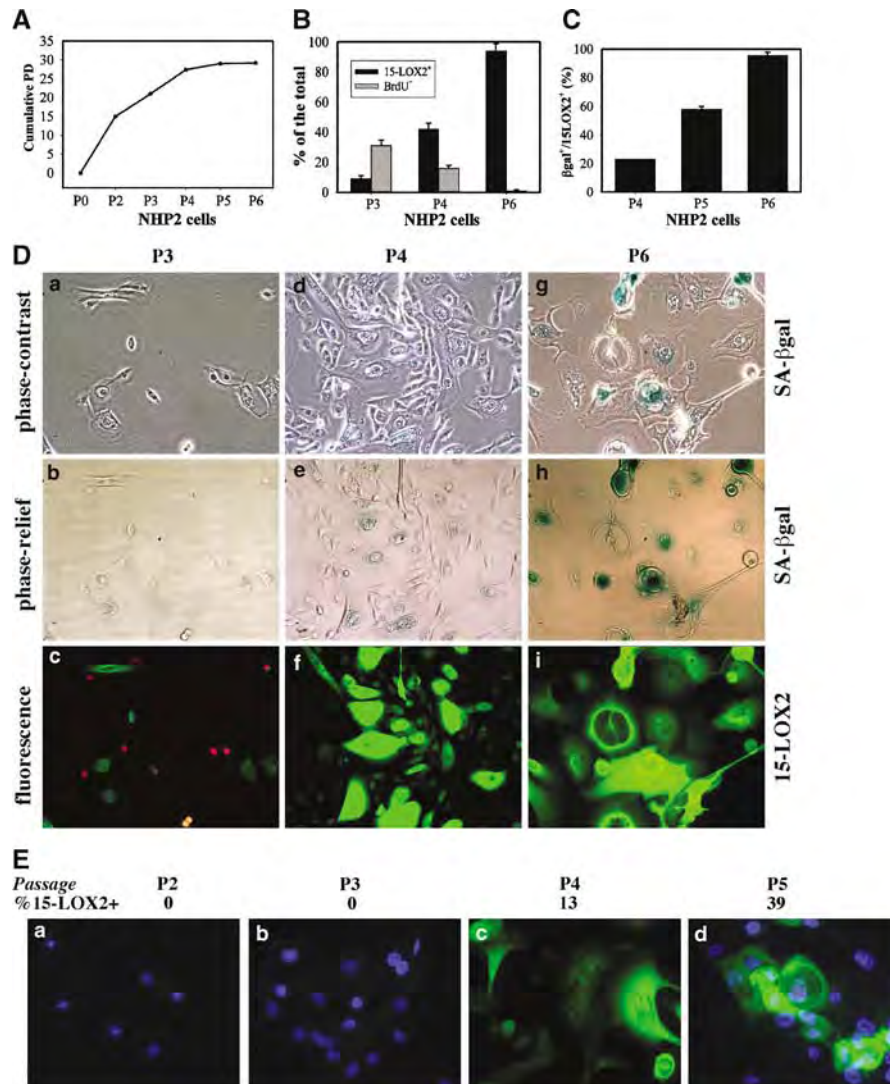some cells (Figure 3Di). As in NHP6 cells, a differential

Figure 3 Induction of 15-LOX2 expression accompanies the replicative senescence of NHP2 (**A–D**) and NHP7 (**E**) cells. (**A**) Cumulative PDs. (**B**) NHP2 cells were processed for BrdU incorporation (4 h pulse) or 15-LOX2 staining. A total of 800–1200 cells was counted and the results are expressed as the mean ± s.e.m. from two experiments. (**C**) Quantification of SA-βgal and 15-LOX2 double-positive cells. (**D**) Representative microphotographs showing NHP2 cells stained for SA-βgal (top and middle panels), BrdU (4 h pulse; red), and 15-LOX2 (green). Original magnifications, ×200. (**E**) Cell-autonomous induction of 15-LOX2 in NHP7 cells. The percentages of 15-LOX2^{+} cells were determined by counting 500–800 cells at each passage. Original magnifications: ×400

counting of the % of SA-βgal^{+} cells in the 15-LOX2^{+} population revealed that only a proportion of the 15-LOX2^{+}-NHP2 cells was SA-βgal[+] (Figure 3C).

Similar induction of 15-LOX2 was observed in NHP7 cells starting from P4 (Figure 3E). The percentages of SA-βgal^{+} cells at P4–P7 were 0, 4, 40, and 75%, respectively, again suggesting that 15-LOX2 induction precedes senescence. Similar induction of 15-LOX2 was also observed in NHP4 cells (not shown).

Since all NHP cells had been constantly cultured in serum/androgen-free and semidefined conditions, these observations suggest that (1) 15-LOX2 is induced in NHP cells in a cell-autonomous manner, (2) 15-LOX2 induction occurs as NHP cells undergo replicative senescence, and (3) 15-LOX2 accumulation precedes cell senescence.

The upregulated 15-LOX2 in NHP cells is enzymatically active

15-LOX2 preferentially metabolizes arachidonic acid (AA) to generate a fatty acid, 15(S)-HETE (Brash *et al.*, 1997; Kilty *et al.*, 1999). To determine whether the upregulated 15-LOX2 in NHP cells is enzymatically active, we measured 15(S)-HETE levels in cultured NHP6 cells in the presence of AA. The results revealed increasing levels of 15(S)-HETE in NHP6 cells as a function of passage (Table 1), suggesting that the induced 15-LOX2 was enzymatically active. The P6 NHP6 cells produced >10 times more of 15(S)-HETE than the P3 NHP6 cells (Table 1). Surprisingly, when the measurement was done in the absence of AA, little 15(S)-HETE was detected in the P6 NHP6 cells

(Table 1). These results suggest that NHP cells normally cultured in the regular serum/androgen-free medium may produce little endogenous 15(S)-HETE. In support, measurement of the 15(S)-HETE levels in the culture media revealed barely detectable amounts (<0.02 ng/medium derived from 10^6 cells) in young (P2) and undetectable amounts in senescent (P7) NHP6 cells.

Transcriptional induction of both 15-LOX2 and its splice variants during NHP cell senescence

To determine whether 15-LOX2 induction resulted from transcriptional activation, we first measured the 15-LOX2 promoter activity in NHP6 cells transfected with the p15LOX2 ($-726/+80$)-luc reporter construct (Tang et al., 2004). As shown in Figure 4B, increasing 15-LOX2 promoter activity was observed

in NHP6 cells with increasing passages. To confirm the promoter assays, we carried out semi-quantitative RT–PCR analysis. As shown in Figure 4C, 15-LOX2 mRNA levels increased as cells underwent senescence. RT–PCR using primers A and B, which detects 15-LOX2 and all its splice variants (Table 1S; Figure 1S; Tang et al., 2002), revealed that the total 15-LOX2 mRNA levels increased ~ 2-fold from P3 to P4 and then further increased (by ~ 5-fold) by P5–P6 (Figure 4C, top).

Previously, using long-distance RT–PCR, we identified three major 15-LOX2 splice variants named 15-LOX2sv-a, 15-OX2sv-b, and 15-LOX2sv-c (Figure 1S; Tang et al., 2002). During the present work, we identified two novel less abundant isoforms, which we named as 15-LOX2sv-d and 15-LOX2sv-e (Figure 4C and D; Figure 1S). 15-LOX2sv-d is identical to 15-LOX2, except that a 45-bp facultative intron in exon 9 (nt1302–nt1346) is spliced out. 15-LOX2sv-e is identical to 15-LOX2sv-c, except for the exon 9 being spliced out. To distinguish 15-LOX2 (i.e., full-length or FL) from its splicing isoforms, we carried out differential RT–PCR using several isoform-specific primers (Figure 4C–E; Table 1S; Figure 1S). The results demonstrated that 15-LOX2 and its splice variants (in particular, 15-LOX2sv-b) all increased, to different levels, in their mRNA levels during NHP cell senescence (Figure 4C–E). Interestingly, the upregulated mRNAs of 15-LOX2 and some of its variants slightly decreased in late-passage NHP6 cells (Figure 4C–E).

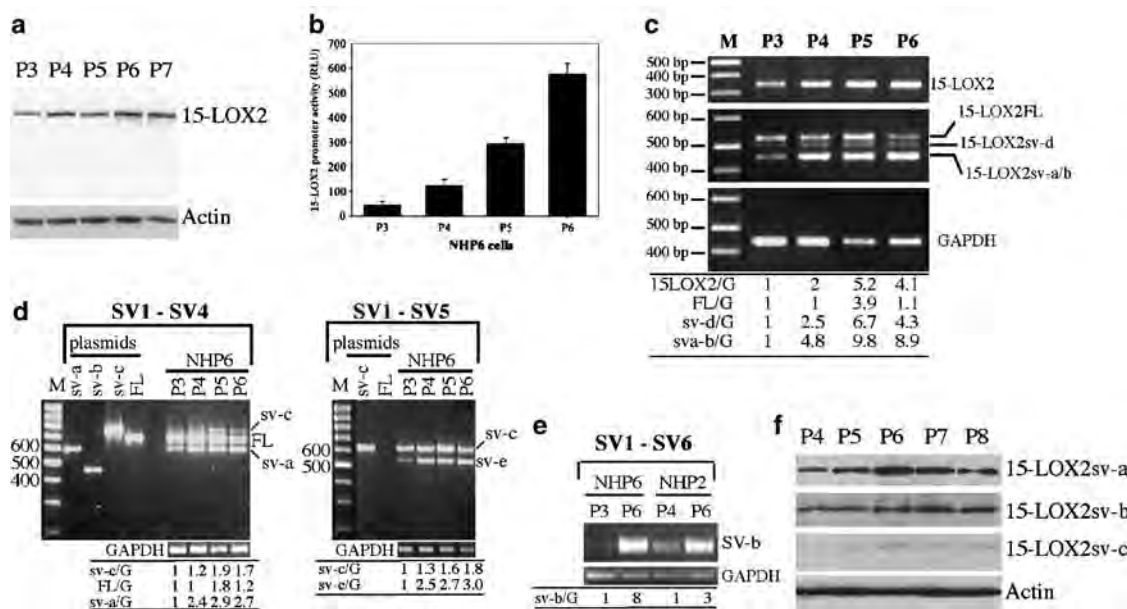The RT–PCR results suggest that as NHP cells underwent senescence, the mRNA levels of both 15-

Table 1 15(S)-HETE production in NHP6 cells of different passages[a]

Passage	15(S)-HETE level (ng/10^6 cells)
Passage 3	1.25 ± 0.23
Passage 4	6.85 ± 0.05*
Passage 5	7.83 ± 0.28*
Passage 6	13.8 ± 2.4**
Passage 6[b]	0.27 ± 0.06

[a]15(S)-HETE production was measured in log-phase cells, in the presence of exogenous AA (100 μM; 37°C $\times$ 10 min) using LC/MS/MS. Data represent the mean $\pm$ s.d. derived from 3–4 samples for each passage. *$P<0.01$ and **$P<0.001$, Student's t-test. [b]15(S)-HETE measurement in the absence of exogenous AA

Figure 4 mRNA and protein induction of 15-LOX2 and its splice variants. (**a**) Western blotting (60 μg/lane) showing increased 15-LOX2 protein expression in passaged NHP6 cells. (**b**) Increased 15-LOX2 promoter activities in passaged NHP6 cells. The results were expressed as the relative luciferase unit (RLU) and the bars represent mean $\pm$ s.e.m. from triplicate samples for each passage. (**c–e**) Increased mRNA levels of 15-LOX2 and its splice variants in NHP6 cells analysed by semiquantitative RT–PCR (see Table S1). The relative mRNA levels of 15-LOX2 or its variants over the corresponding GAPDH (**G**) mRNAs were determined by densitometric scanning. (**f**) 15-LOX2 splice variants were upregulated during NHP cell senescence. In all, 60 μg of NHP6 cell lysate was used in Western blotting using the newly generated peptide antibodies against individual 15-LOX2 isoforms

LOX2 and its splice variants were induced, leading to an increase in 15-LOX2 protein(s) detected on immuno-fluorescence. On Western blotting, the same rabbit polyclonal anti-15-LOX2 antibody also detected increased 15-LOX2 protein (Figure 4A). However, for unknown reasons, this antibody did not recognize the 15-LOX2 splice variants well on Western blotting (Figure 4A), as previously observed (Tang et al., 2002; Bhatia et al., 2003). To circumvent this problem, we made isoform-specific peptide polyclonal antibodies. Using these antibodies, we examined the protein levels of three major 15-LOX2 splice variants in passaged NHP6 cells. As shown in Figure 4F, 15-LOX2sv-a increased at P5, reached the peak level at P6, and then slightly decreased at P7-P8. By contrast, 15-LOX2sv-b continued to increase as a function of cell passage (Figure 4F). 15-LOX2sv-c showed similar changes as 15-LOX2sv-b although its expression levels were lower than those of 15-LOX2sv-b (Figure 4F).

Stable expression of 15-LOX2 or 15-LOX2sv-b in PC3 PCa cells results in a passage-related, senescence-like phenotype

In the following experiments, we attempt to determine whether 15-LOX2 induction causally contributes to NHP cell senescence. We first examined by Western blotting 15-LOX2 expression in both newly established and long-term cultured PCa cell lines (> 15) as well as in several pairs of prostate epithelial cells immortalized by either viral oncogenes (i.e., SV40 large T antigen, HPV18, or E6/E7) or the catalytic subunit of human telomerase (hTERT) and their preimmortalized counterparts. We did not detect 15-LOX2 expression in any of the immortalized prostate epithelial or PCa cells (not shown), consistent with some of our earlier results (Tang et al., 2002). These observations suggest that 15-LOX2 expression is inversely correlated with cell immortality.

Next, we followed PC3 cells that had been stably transfected with 15-LOX2 or 15-LOX2sv-b (Bhatia et al., 2003) for multiple passages. These cells were derived from clonal cultures and enough cells generally became available for analysis only at P4–P5. To our surprise, these cells also showed passage-related phenotypic changes resembling replicative senescence in NHP cells. For instance, most of the early-passage (i.e., P3–P4) cells were generally small, actively proliferating, and SA-βgal-negative (not shown). By P6, 10–15% of the stably transfected cells became big and flat, some of which were also SA-βgal$^+$ (Figure 5a and b) and most of which were BrdU$^-$ (not shown). With increasing passage, the percentage of big/flat cells also increased in both 15-LOX2- and 15-LOX2sv-b-expressing PC3 cells (Figure 5a and b).

Enforced expression of 15-LOX2 or 15-LOX2sv-b in young NHP7 cells by retroviral infection also induces cell-cycle arrest and a senescence-like phenotype

Next, we carried out gain-of-function experiments in young NHP cells by taking advantage of the fact that

NHP7 cells at P2 and P3 do not express 15-LOX2 and $\sim$14% of the cells start expressing 15-LOX2 at P4 (Figure 3E). Using two pBabe15LOX2-EGFP and two pBabe15LOX2sv-b-EGFP vectors and the pBabe-EGFP as control, we infected P2 NHP7 cells cultured at clonal density (i.e., 1000 cells/T25 slide flask). Triplicate slide flasks were terminated 1 week after infection and analysed for 15-LOX2 expression, BrdU incorporation, SA-βgal positivity, and morphological changes. As shown in Table 2 and Figure 6a, 82–96% of the P2 NHP7 cells were infected with the GFP-tagged retroviral vectors and the majority of the infected GFP$^+$ cells were positive for 15-LOX2. No 15-LOX2$^+$ cells were observed in uninfected or pBabe-EGFP-infected flasks (Table 2; Figure 6a). Some (8–22%) GFP$^+$ cells did not express 15-LOX2 (Table 2), perhaps because the retroviral LTR promoter was silenced. Interestingly, a very small percentage of (1–3%; Table 2) GFP$^-$ cells were 15-LOX2$^+$, possibly due to the inactivation of the pCMV promoter in these cells.
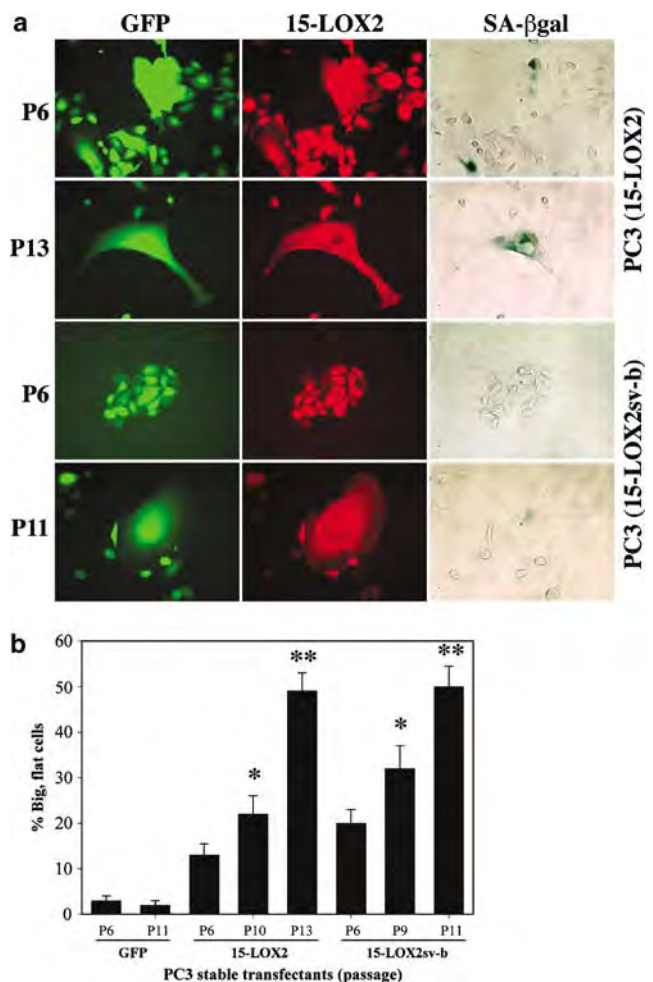

Figure 5 Stable overexpression of 15-LOX2 in PCa cells induces premature senescence. (**a**) PC3 cells stably transfected with 15-LOX2 or 15-LOX2sv-b at different passages were stained for 15-LOX2 (middle panels) and SA-βgal (right panels). (**b**) Quantification of senescence-like (big and flat) cells. *$P<0.05$; **$P<0.01$ (Student's t-test)

Table 2 15-LOX2 expression in NHP7 (P2) cells infected with retroviral expression vectors[a]

Cells	GFP+		GFP−	
	15-LOX2+	*15-LOX2−*	*15-LOX2+*	*15-LOX2−*
Uninfected	0	0	0	100%
pBabe-EGFP	0	67%	0	33%
pBabe15LOX2-EGFP (clone 3)	75%	8%	1%	16%
pBabe15LOX2-EGFP (clone 4)	73%	9%	3%	15%
pBabe15LOX2sv-b-EGFP (clone 1)	81%	11%	1%	8%
pBabe15LOX2sv-b-EGFP (clone 2)	74%	22%	0	4%

[a]NHP7 (P2) cells were plated in triplicate at clonal density (1000 cells/T25 slide flask) and either uninfected or infected with the indicated retroviral vectors. After 1 week, cells were fixed and processed for 15-LOX2 immunostaining. On average 200–300 cells were counted for each condition

When GFP+/15-LOX2+ and GFP+/15-LOX2− NHP7 cells at P2 were compared at 7 days post infection for their morphology, many more of the GFP+/15-LOX2+ cells were big and flat (Figure 6a). Quantitative analyses revealed that both clones, each of 15-LOX2 and 15-LOX2sv-b retroviral vectors, increased the percentage of big and flat cells (Figure 6d). Infection with GFP-encoding retroviral vector also slightly increased the percentage of big and flat cells although the differences were not statistically significant (Figure 6d). As observed in stably transfected PC3 cells (Figure 6), only some of these big and flat cells stained strongly for SA-βgal (not shown). When GFP+ and GFP− NHP7 cells at P2 were compared, at 7 days post infection, for BrdU incorporation, significantly more GFP− cells were found to be BrdU+ (Figure 6b and c). Since the majority of GFP+ cells were 15-LOX2+ (Table 2), these results suggest that enforced 15-LOX2 expression in young NHP7 cells inhibits cell proliferation. Enforced expression of 15-LOX2sv-b similarly decreased BrdU+ cells (Figure 6c) and increased the percentage of big and flat cells (Figure 6a and d).

Next, we asked how enforced 15-LOX2 expression might affect the long-term proliferation of young NHP7 cells. We similarly infected the clonally cultured P2 NHP7 cells with various retroviral vectors and followed these cells for 5 weeks. As shown in Figure 7, by 5 weeks, the initially plated 1000 cells either uninfected (a–e) or infected with pBabe-EGFP (f–j) proliferated extensively resulting in nearly confluent cultures. Little or only faint 15-LOX2 expression was detected in these cells (Figure 7c and h). By contrast, NHP7 cells infected with pBabe15-LOX2 only marginally increased in cell number and most of the infected cells were 15-LOX2+ with enlarged and flattened morphology (Figure 7k–o). Surprisingly, NHP7 cells infected with pBabe15-LOX2sv-b, which initially behaved like the cells infected with the pBabe15LOX2-EGFP, gradually picked up proliferation and eventually resulted in confluent cultures (Figure 7p–t). Most of these cells were GFP+ although a significant percentage of the cells was GFP− (Figure 7t). 15-LOX2 staining revealed that the majority of these cells had lost 15-LOX2 expression (Figure 7r) and only a few of the 15-LOX2+, big and flat cells could be observed (not shown). Several replicate experiments with triplicate flasks infected with two clones each of 15-

LOX2 or 15-LOX2sv-b revealed similar results (Figure 7 and data not shown).

15-LOX2 expression in human prostate appears to correlate with age

To determine whether 15-LOX2 expression *in vivo* might be associated with age, we carried out a pilot immuno-histochemical survey of 15-LOX2 staining in human prostate tissues of various ages. As a control, these samples were also stained for PSA, an androgen-regulated gene. Both 15-LOX2 and PSA were negative in infant prostate tissues (not shown; $n = 2$). In three samples of 15-year-old prostate, 15-LOX2 staining (Figure S2A) was negative although PSA staining was clearly positive (Figure S2B). 15-LOX2 staining became focally positive at age 18 (Figure S2C) and then significantly increased in adult prostates (Figure S2E; $n = 3$). In the prostates of ≥50 years ($n = 2$), 15-LOX2 staining became homogeneously strong (Figure S2G). In the lumens of adult prostatic glands, 15-LOX2-positive secretions were easily observed (Figure S2E and G, arrows). In contrast, the PSA showed overall similar staining patterns and intensities in the ≥18-year-old prostates (Figure S2D, F and H).

Discussion

The main goal of the present study was to study molecular mechanisms associated with the senescence of NHP cells. Our immunophenotyping experiments reveal that all young primary NHP cells are CK5+/CK18+ intermediate basal cells that also express p63, hTERT, CD44, and α2β1, molecules proposed to mark prostate stem/progenitor cells (Liu *et al*., 1997; Signoretti *et al*., 2000; Collins *et al*., 2001). When serially cultured in the semidefined medium containing EGF and insulin, NHP cells gradually lose their proliferative potential and progenitor markers, suggesting that the simple culture conditions used here are insufficient to maintain the progenitor cell properties of the NHP cells. Interestingly, in a similar chemically defined medium containing PDGF and insulin, most perinatal rat oligodendrocyte precursor cells seem to be able to proliferate for years without losing progenitor markers (Tang *et al*., 2000,
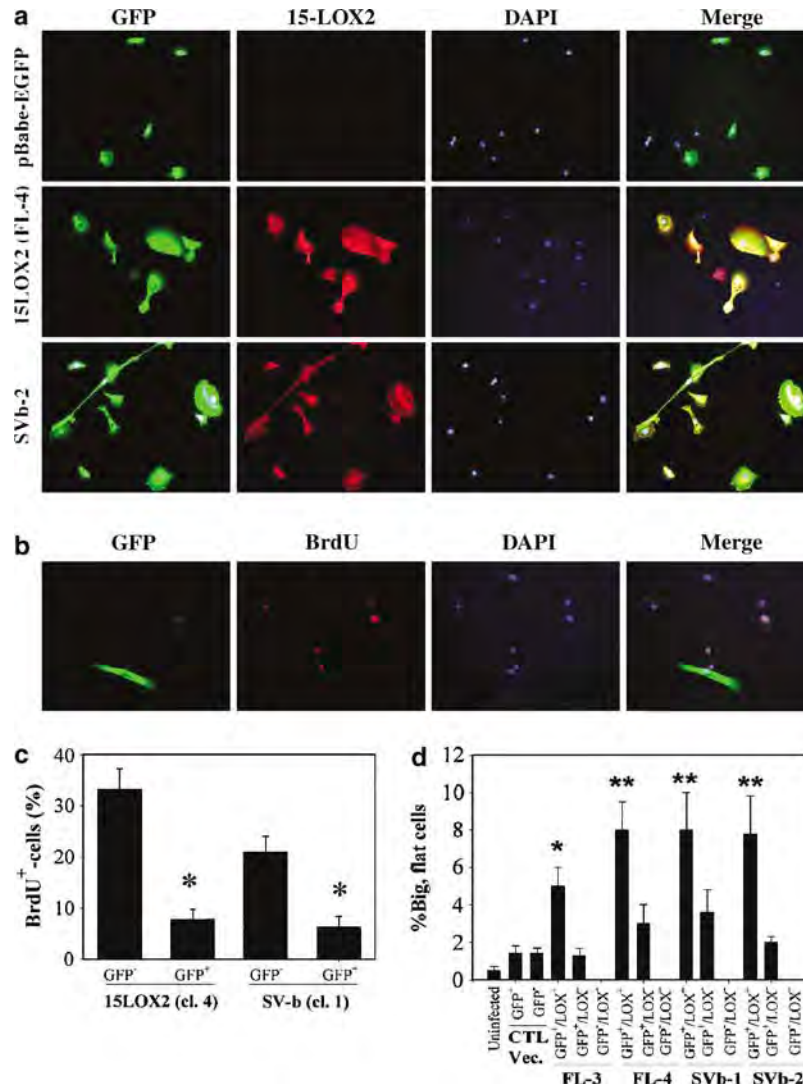
Figure 6 Enforced expression of 15-LOX2 or 15-LOX2sv-b in early-passage NHP7 cells induces cell-cycle arrest and a senescence-like phenotype. NHP7 cells at P2 were plated at clonal density were either untransduced or infected with pBabe-EGFP, pBabe15LOX2-EGFP (two clones used, that is, FL-3 and FL-4; see Table 2), or pBabe15LOXsv-b-EGFP (two clones used, that is, SVb-1 and SVb-2; see Table 2). After 1 week, cells were processed for 15-LOX2 (**a**), BrdU (**b**), or SA-βgal (not shown) staining. Representative images from one clone each of 15-LOX2 or 15-LOX2sv-b-infected cultures were shown. Original magnifications: $\times 200$. (**c**) The % of BrdU$^+$ cells was determined in the GFP$^+$ or GFP$^-$ population. The results represent the mean $\pm$ s.d. *$P < 0.01$. (**d**) % (mean $\pm$ s.d.) of big and flat cells. *$P < 0.01$; **$P < 0.001$

2001). It is unclear at the moment whether the different behaviors of these two cell types are caused by differences in cell lineages, initiating cell ages, or species.

Cultured NHP cells generally undergo a total of 23–30 PDs and their proliferative lifespan does not seem to be correlated with donor ages. For example, NHP7 cells derived from a 14-year-old donor undergo ~ 23 PDs, whereas NHP2 cells derived from a 59-year-old donor undergo ~ 30 PDs. This lack of correlation between donor age and cumulative PDs of NHP cells resembles that in human fibroblasts (Cristofalo *et al.*, 1998). NHP cells undergo replicative senescence evidenced by cessation of proliferation, loss of progenitor properties, enlarged and flattened morphology, and expression of SA-βgal. Both presenescent and fully senescent NHP

cells in culture do not acquire any differentiation markers such as AR, PSA, and PAP, molecules expressed mainly in the lumenal cells. Strikingly, 15-LOX2, another lumenal cell-specific molecule, is induced accompanying cell senescence under the non-differentiating culture conditions. Both 15-LOX2 and its splice variants are induced and the induction takes place in a cell-autonomous manner at the transcription level. How 15-LOX2 gene transcription is activated remains to be investigated although it does not seem to involve androgen/AR pathway as there is no androgen in the medium and NHP cells have always been AR-negative at the protein level. Moreover, androgen/AR pathway does not directly regulate the 15-LOX2 gene expression in NHP cells (Tang *et al.*, 2004). One possibility is
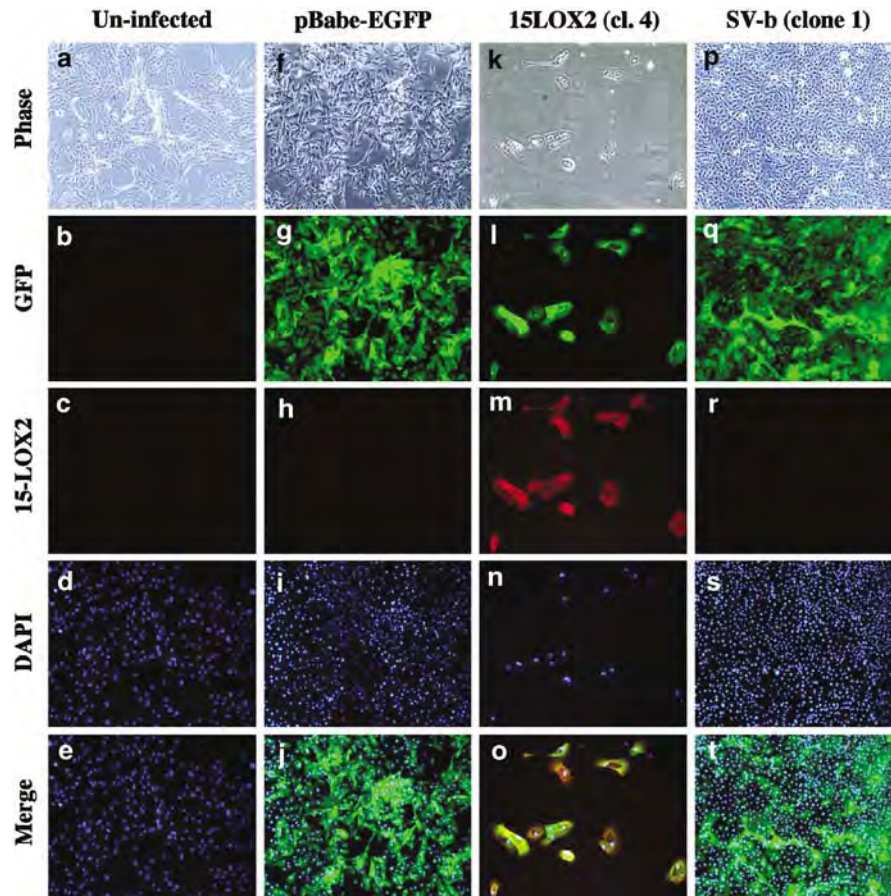
Figure 7 Enforced expression of 15-LOX2 inhibits long-term proliferation of NHP7 cells. NHP7 cells at P2 were infected as detailed in Figure 6 legend. Cells were processed for 15-LOX2 staining 5 weeks after infection. Representative images from one clone each of 15-LOX2 or 15-LOX2sv-b-infected cultures were shown. Original magnification: × 100

through increased Sp1 transcriptional activity (Tang et al., 2004). In support, 15-LOX2 promoter constructs with the Sp1 sites mutated, when transfected into late-passage NHP6 cells, possess much lower promoter activities compared to the constructs with the intact Sp1 sites (Tang et al., 2004).

Several pieces of evidence suggest that induction and accumulation of 15-LOX2 and its splice variants may contribute to NHP cell-cycle arrest and senescence. First, 15-LOX2 expression is inversely correlated with NHP cell proliferation. Second, 15-LOX2 expression is inversely correlated with cell immortality. Third, 15-LOX2 behaves as a functional prostate tumor suppressor in that the 15-LOX2 mRNA, protein, and enzymatic activity are decreased or lost in PCa (Shappell et al., 1999) and re-expression of 15-LOX2 inhibits PCa cell proliferation in vitro and tumor development in vivo (Tang et al., 2002). Fourth, 15-LOX2 induction precedes the cell-cycle arrest and onset of NHP cell senescence. Fifth, remarkably, stable re-expression of 15-LOX2 in PCa cells, which apparently have bypassed the senescence checkpoint, also leads to a senescence-like phenotype. Finally, enforced expression of 15-LOX2 in young NHP cells results in a senescence-like phenotype.

It is worth pointing out that enforced expression of 15-LOX2 in either young NHP or PCa cells does not seem to lead to a full manifestation of senescence as only a fraction of the cells is arrested in cell cycle and becomes big and flat and most of these big and flat, presumably senescent cells only stain weakly for SA-βgal. These results, which are not surprising, suggest that 15-LOX2 may represent only one of the multiple factors required to cause permanent cell-cycle exit and full senescence in NHP cells.

How does 15-LOX2 upregulation contribute to NHP cell senescence? As NHP cells undergo senescence, they upregulate cyclin-dependent kinase inhibitors (CKIs) p16 and p21. The downstream target of p16, pRb, as well as the upstream activator of p21, p53, are also upregulated (Bhatia et al., unpublished observations). Together, their concerted action may arrest NHP cells in the G1 phase of the cell cycle. How may 15-LOX2 expression contribute to CKI upregulation and cell cycle arrest? One possibility may be via its product, 15(S)-HETE, which has been proposed as a ligand PPAR-γ (Huang et al., 1999), the latter of which in turn may inhibit cell-cycle progression by inhibiting cyclin D1 (Wang et al., 2001). Although the 15-LOX2/15(S)-

HETE/PPARγ pathway cannot be excluded, several pieces of evidence suggest that the 15-LOX2-associated NHP cell senescence might not depend on AA metabolism and 15(S)-HETE production. *First*, although the induced 15-LOX2 is enzymatically active, late-passage/senescent NHP cells do not produce more endogenous 15(S)-HETE than the young NHP cells, nor do they secrete 15(S)-HETE into the medium. *Second*, although exogenous 15(S)-HETE can cause cell-cycle arrest in NHP or PCa cells, it does so only at high (i.e., μM) concentrations and without inducing a senescence-like phenotype (Tang *et al*., 2002 and unpublished observations). *Third*, not only 15-LOX2 but also its splice variants are induced during NHP cell senescence. Most 15-LOX2 splice variants either have much reduced (e.g., for 15-LOX2sv-a; Kilty *et al*., 1999) or completely lack (e.g., 15-LOX2sv-b and 15-LOX2sv-c; Bhatia *et al*., 2003), the AA-metabolizing activity and do not produce appreciable amounts of 15(S)-HETE. *Fourth*, just as 15-LOX2sv-b possesses tumor-inhibitory effects (Bhatia *et al*., 2003), enforced expression of 15-LOX2sv-b in young NHP or PCa cells also induces cell-cycle arrest and a senescence-like phenotype. Interestingly, most NHP cells infected with 15-LOX2sv-b retroviral vectors lose the transgene expression by 5 weeks, proliferate fast, and populate the culture dishes. The underlying mechanisms remain to be determined.

These discussions support a dual-action model in which 15-LOX2 and its splice variants possess both AA-dependent and AA metabolism-independent biological activities (Bhatia *et al*., 2003). Another mammalian LOX, 15-LOX1, is well known to bind biological membranes and catalyse their degradation independently of fatty acid metabolism (Kuhn and Borngraber, 1999). 15-LOX2 also shows significant membrane-binding capacities (Bhatia *et al*., 2003). Our preliminary data suggest that 15-LOX2 accumulation results in alterations in organelle membranes and increased oxidative stress, consistent with the prominent cytoplasmic vacuoles in senescent NHP cells. Oxidative stress in NHP cells may theoretically trigger telomere attrition and deprotection leading to cell-cycle checkpoint responses and subsequent senescence.

In summary, we have presented evidence that 15-LOX2 is involved in NHP cell senescence in culture. Although the *in vivo* biological relevance of this finding with regard to prostate aging remains to be determined, it is of interest that 15-LOX2 expression appears to increase with age, suggesting that 15-LOX2 might represent an endogenous prostate senescence gene. This possibility is consistent with 15-LOX2 being expressed only in a limited number of human tissues (i.e., prostate, lung, hair root, and cornea) and expressed most abundantly in prostate. Studies in multiple NHP cells suggest a direct correlation between the 15-LOX2 expression levels and the senescence phenotype, that is, cells that are strongly 15-LOX2-positive also show a fully senescent phenotype: big/flat morphology, lack of progenitor markers, and SA-βgal-positive. These observations in NHP cells, together with the 15-LOX2-induced senescence phenotype in PCa cells, suggests that

15-LOX2 expression, or the chronic damage induced by 15-LOX2, may have to accumulate to a certain threshold to help trigger cell senescence and that *in vivo* the relative low levels of 15-LOX2 in young prostate may play a differentiation-related function, whereas accumulated 15-LOX2 or 15-LOX2-induced cellular damages in older prostate may contribute to the senescence and aging phenotype. As the biological essence of replicative senescence is cell-cycle arrest and senescence has been considered a critical barrier to acquisition of immortality and tumorigenic transformation, the results presented herein provide novel mechanistic insight on (1) the normal developmental history of prostate stem/progenitor cells, (2) molecular mechanisms underlying NHP cell senescence, and (3) how 15-LOX2 may suppress tumor development and why its expression is shut down in PCa cells.

Materials and methods

Cells and reagents

NHP2, NHP4, and NHP6 cells (Tang *et al*., 2002, 2004) and NHP7 cells (Clonetics) were cultured on collagen-coated dishes in serum- and androgen-free, PrEBM medium supplemented with insulin, EGF, hydrocortisone, bovine pituitary extract, and cholera toxin, and used at passage 2–8. Luciferase reporter plasmids and anti-15-LOX2 antibody were previously described (Bhatia *et al*., 2003; Tang *et al*., 2004). Other antibodies used in this study include: polyclonal anti-CK5 (BabCO), monoclonal anti-CK18 (clone RGE53; BD PharMingen), monoclonal anti-p63 (clone 4A4), PSA and AR (Santa Cruz), 2 monoclonal anti-CD44 (clone G44-26 from BD PharMingen and DF1485 from Santa Cruz), polyclonal anti-hTERT (AbCAM), monoclonal anti-CD57 (clone NK-1) and $\alpha2\beta1$ (PharMingen), and monoclonal anti-PAP (clone PASE/4LJ; Dako). Secondary antibodies were acquired from Amersham. 15-HETE and 15-HETE-d_8 were from Cayman. AA and butylated hydroxytulene (BHT) were obtained from Sigma.

Immunofluorescence

Basic procedures were described (Tang *et al*., 2002; Bhatia *et al*., 2003). For cytoskeletal proteins (i.e., CK5 and CK18), cells were fixed and permeabilized in methanol/acetone (1:1; -20°C) for 10 min and then used in immunostaining. Cells were both analysed for fluorescence intensity and quantified for % positive cells. For the latter, 600–1200 cells were counted for each condition. Statistical analyses were performed using Student's *t*-test.

Semi-quantitative RT–PCR

Total RNA was isolated from NHP cells and used in RT–PCR as detailed in Table 1S.

Cumulative BrdU labeling and determination of PDs

Cumulative PDs were determined using a modified 3T3 protocol. For cumulative BrdU labeling (Tang *et al*., 2000, 2001), NHP cells were pulsed with 10 μM BrdU for 2–120 h, fixed in 4% paraformaldehyde, permeabilized in 70% ethanol (in PBS, -20°C) for 10 min, and then incubated with monoclonal anti-BrdU antibody followed by goat anti-mouse IgG-Rhodamine. Finally, cells were nuclear counterstained

with DAPI. A total of 1000–1200 cells were counted per coverslip to determine the proportion of BrdU$^+$ cells. The labeling index was plotted against the BrdU pulse time to obtain a cumulative labeling curve. PD was determined using the formula $2^x = N_f/N_i$, where x is the PD, N_f is the final cell number and N_i is the number of cell initially plated. The approximate PD time was determined using the formula $d = t/\log_2 N$, where d is the PD time, t is the time of cells in culture, and N is the total number of cells.

Western blotting

Whole cell lysates were used in Western blotting using ECL (Tang *et al.*, 2002; Bhatia *et al.*, 2003).

Senescence-associated β-galactosidase (SA-βgal) staining

NHP cells of different passages were stained for SA-βgal (Dimri *et al.*, 1995; Tang *et al.*, 2001). In some experiments, triple staining of 15-LOX2, BrdU, and SA-βgal was carried out.

15-HETE measurement in NHP cells or culture medium by liquid chromatography and tandem mass spectrometry (LC/MS/MS)

15(S)-HETE levels in NHP cells were measured as previously detailed (Tang *et al.*, 2002). Eicosanoids in culture medium were measured using a solid-phase method and the detailed protocol is available upon request.

Preparation of 15-LOX2 isoform-specific peptide antibodies

Peptide sequences at the splicing junctures (Tang *et al.*, 2002) were utilized as immunogens to produce isoform-specific antibodies. Specifically, peptides YRDDGMQIWGIPSSLE (for 15-LOX2sv-b), HHPPPKAWQHARAS (for 15-LOX2sv-c), and HPLFKSTGIGIEGF (for 15-LOX2sv-a) were chemically synthesized by coupling to an N-terminal cysteine, HPLC purified, and then utilized to immunize the New Zealand White rabbit by intradermal injection. The antibodies produced were affinity-purified using the commercial Kit (Pierce, Rockford, IL, USA). The purified antibodies, together with the preimmune sera were characterized using

PCa cells transfected with the individual cDNAs (Bhatia *et al.*, 2003).

Retroviral experiments

We made several bi-cistronic pBabe-EGFP retroviral vectors in which 15-LOX2 or 15-LOX2sv-b is driven by the viral LTR, whereas GFP is expressed from the CMV promoter. 15-LOX2 or 15-LOX2sv-b cDNA was released from pCMS-EGFP-15LOX2 or pCMS-EGFP-15LOX2sv-b vectors (Bhatia *et al.*, 2003) and the cDNAs were ligated into pBabe-EGFP (Tang *et al.*, 2001) retroviral vector. Two 15-LOX2 and two 15-LOX2sv-b retroviral vectors were transfected into the Ampho-Phoenix packaging cells using FuGENE 6. At 48 h post transfection, viral particles were collected from the culture medium by ultracentrifugation and used to infect NHP cells (Tang *et al.*, 2001).

Immunohistochemical staining of 15-LOX2 and PSA

Paraffin-embedded sections of archival prostate tissues of different ages, including infant (one 2-month-old, one 1-year-old), adolescent (three 15-year-old and one 18-year-old), adult (one case each of 36-, 38-, and 43-year-old), and senior (three 50-year-old and two 62-year-old), were used in staining for 15-LOX2 and PSA. Tissue sections were incubated with 3% H_2O_2 to block endogenous peroxidase activity and in 10 mM citrate buffer (pH 6.0) for 10 min in a microwave oven for antigen retrieval. Slides were then incubated in 10% goat whole serum in PBS for 30 min to block nonspecific binding and then in anti-15-LOX2 antibody. Finally, slides were incubated with anti-rabbit HRP (30 min at room temperature) and then with the substrate DAB.

References

Bhatia B, Maldonado CJ, Tang S, Chandra D, Klein RD, Chopra D, Shappell SB, Yang P, Newman RA and Tang DG. (2003). *J. Biol. Chem.*, **278**, 25091–25100.

Bonkhoff H, Stein U and Remberger K. (1994). *Prostate*, **24**, 42–46.

Brash AR, Boeglin WE and Chang MS. (1997). *Proc. Natl. Acad. Sci. USA*, **94**, 6148–6152.

Castro P, Giri D, Lamb D and Ittman M. (2003). *Prostate*, **55**, 30–38.

Choi J, Shendrik I, Peacocke M, Peehl D, Buttyan R, Ikeguchi EF, Katz AE and Benson MC. (2000). *Urology*, **56**, 160–166.

Collins AT, Habib FK, Maitland NJ and Neal DE. (2001). *J. Cell Sci.*, **114**, 3865–3872.

Cristofalo VJ, Allen RG, Pignolo RJ, Martin BG and Beck JC. (1998). *Proc. Natl. Acad. Sci. USA*, **95**, 10614–10619.

Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, Peacocke M and Campisi J. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 9363–9367.

Garraway LA, Lin D, Signoretti S, Waltregny D, Dilks J, Bhattacharya N and Loda M. (2003). *Prostate*, **55**, 206–218.

Hanahan D and Weinberg RA. (2000). *Cell*, **100**, 57–70.

Huang JT, Welch JS, Ricote M, Binder CJ, Wilson TM, Kelly C, Witztum JL, Funk CD, Conrad D and Glass CK. (1999). *Nature*, **400**, 378–382.

Hudson DL, O'Hare M, Watt FM and Masters JRW. (2000). *Lab. Invest.*, **80**, 1243–1250.

Jarrard DF, Sarkar S, Shi Y, Yeager TR, Magrane G, Kinoshita H, Nassif N, Meisner L, Newton MA, Waldman FM and Reznikoff CA. (1999). *Cancer Res.*, **59**, 2957–2964.

Kilty I, Alison L and Vickers PJ. (1999). *Eur. J. Biochem.*, **266**, 83–93.

Kinbara H, Cunha GR, Boutin E, Hayashi N and Kawamura J. (1996). *Prostate*, **29**, 107–116.

Kuhn H and Borngraber S. (1999). *Adv. Exp. Med. Biol.*, **447**, 5–28.

Liu AY, True, LD, LaTray, L, Nelson PS, Ellis WJ, Vessella, RL, Lange, PH, Hood L and van den Engn G. (1997). *Proc. Natl. Acad. Sci. USA*, **94**, 10705–10710.

Raff M. (2003). *Annu. Rev. Cell Dev. Biol.*, **19**, 1–22.

Richardson GD, Robson CN, Lang SH, Neal DE, Maitland NJ and Collins AT.. (2004). *J. Cell Sci.* (E-pub June 29).

Robinson EJ, Neal DE and Collins AT. (1998). *Prostate*, **37,** 149–160.

Ruijter E, van de Kaa C, Miller G, Ruiter D, Debruyne F and Schalken J. (1999). *Endocr. Rev.*, **20,** 22–45.

Sandhu C, Peehl DM and Singerland J. (2000). *Cancer Res.*, **60,** 2616–2622.

Schalken JA and van Leenders G. (2003). *Urology*, **62,** 11–20.

Schmitt CA, Fridman JS, Yang M, Lee S, Baranov E, Hoffman RM and Lowe SW. (2002). *Cell*, **109,** 335–346.

Schwarze SR, Shi Y, Fu VX, Watson PA and Jarrard DF. (2001). *Oncogene*, **20,** 8184–8192.

Shappell SB, Boeglin WE, Olson SJ, Kasper S and Brash AR. (1999). *Am. J. Pathol.*, **155,** 235–245.

Shou J, Ross S, Koeppen H, de Sauvage FJ and Gao W-Q. (2001). *Cancer Res.*, **61,** 7291–7297.

Signoretti S, Waltregny D, Dilks J, Isaac B, Lin, D, Garraway L, Yang A, Montironi R, McKeon F and Loda M. (2000). *Am. J. Pathol.*, **157,** 1769–1775.

Tang DG, Tokumoto YM and Raff MC. (2000). *J. Cell Biol.*, **148,** 971–984.

Tang DG, Tokumoto YM, Apperly JA, Lloyd AC and Raff MC. (2001). *Science*, **291,** 868–871.

Tang S, Bhatia B, Maldonado C, Yang P, Newman RA, Liu J, Chandra D, Traag J, Klein RD, Fischer SM, Chopra D, Shen J, Zhau H, Chung LW-K and Tang DG. (2002). *J. Biol. Chem.*, **277,** 16189–16201.

Tang S, Bhatia B, Zhou J-J, Maldonado CJ, Chandra D, Kim E, Fischer S, Butler AF, Friedman SL and Tang DG. (2004). *Oncogene*, **23,** 6942–6953.

Tran CP, Lin C, Yamashiro J and Reiter RE. (2002). *Mol. Cancer Res.*, **1,** 113–121.

Untergasser G, Koch, HB, Menssen A and Hermeking H. (2002). *Cancer Res.*, **62,** 6255–6262.

van Leenders G, Dijkman H, van de Kaa H, Ruiter D and Schalken J. (2000). *Lab. Invest.*, **80,** 1251–1258.

Wang C, Fu M, A'Mico M, Albanese C, Zhou JN, Brownlee M, Lisanti MP, Chatterjee VKK, Lazar MA and Pestell RG. (2001). *Mol. Cell. Biol.*, **21,** 3057–3070.

Wright WE and Shay JW. (2001). *Curr. Opin. Genet. Dev.*, **11,** 98–103.

Supplementary Information accompanies the paper on Oncogene website (http://www.nature.com/onc)

table S1. PCR primers and conditions used to detect 15-LOX2 and its splice variants

Name	Sequence[a]	Location[b]
A/B[c]	(A) sense, 5'-AACTCACCCCCACCACCATACACA-3' (B) antisense, 5'-TTCCCGCCTCCATCTCCCA AAGT-3'	nt2234-2584
C/D[d]	(C) sense, 5'-ACTACCTCCCAA AGAACTTCCCC-3' (D) antisense, 5'-TTCAATG CCGATGCCTGTG-3'	nt835-1379
SV1[e]	sense, 5'-ACATCTGCTGCCTGAGGTCTTCAC-3'	nt1204-1226
SV2	sense, 5'-GCTACTACTACCGTGATGATGGGA-3'	nt1481-1504
SV3	antisense, 5'-CTCAGGCAGACACAGGAGAGAATAG-3'	nt1422-1436
SV4	antisense, 5'-ATGTGGCATTGACAGGTGGGAG-3'	nt1848-1873
SV5	antisense, 5'-GAATGAGGCTGAGGAGCTGGG-3'	nt1794-1814
SV6	antisense, 5'-GAGGAGGGTATACCCCAGATCTGC-3'	splicing junction of exons 9/10

[a]The 15-LOX2 sequence was based on Brash et al., 1997 and Tang et al., 2002 (GenBank accession numbers U78294 and AF468051-AF468054).

[b]The nucleotide (nt) locations of the PCR primers were based on the original numbering system in Brash et al., 1997.

[c]This pair of primers detects 15-LOX2 and all splice variants (fig S1). The conditions were: 94ºC x 30s, 60ºC x 45s, and 72ºC x 1 min for a total of 29 cycles.

[d]This pair of primers detects 15-LOX2, 15-LOX2sv-a/sv-b together, and 15-LOX2sv-e (fig. S1). The conditions were: 94ºC x 30s, 60ºC x 1 min, and 72ºC x 1 min for a total of 34 cycles.

[e]The combination of SV1 and SV3 is similar to primers C-D (fig. S1). The conditions were: 94ºC x 30s, 60ºC x 45s, and 72ºC x 1 min for a total of 30 cycles.

The combination of SV1 and SV4 should detect 15-LOX2 and 15-LOX2sv-a/b/c (fig. S1). The conditions were: 94ºC x 1 min, 60ºC x 1 min, and 72ºC x 1 min for a total of 34 cycles.

The combination of SV1 and SV5 detects 15-LOX2sv-c and 15-LOX2sv-e (fig. S1). The conditions were: 94ºC x 45s, 60ºC x 45s, and 72ºC x 1 min for a total of 30 cycles.

The combination of SV1 and SV6 detects specifically 15-LOX2sv-b (fig. S1). The conditions were: 94ºC x 30s, 60ºC x 45s, and 72ºC x 1 min for a total of 30 cycles.

*For reverse transcription (RT), 2 μg of freshly purified total RNA from NHP cells was incubated (42ºC x 2 hr) in a total of 20 μl reaction containing random hexomers and Superscript II reverse transcriptase. For PCR, 2 μl of cDNA was used in a 25 μl reaction containing 0.5 μM primers, dNTPs, and Taq, All PCR reactions were optimized by multiple initial gradient and/or touch-down PCR analysis and shown to amplify most or all intended products at the exponential phase.

FIGURE LEGENDS

figure 1S. *Schematic of 15-LOX2 splicing isoforms*. The exon-intron organization, splicing events, and PCR primers (also see *table S1*) are illustrated. The numbering system is based on Brash *et al*. (1997) with the translational ATG starting at nucleotide (nt) 72 in exon 1. The first three major splice variants, i.e., 15-LOX2sv-a/b/c, were previously described (Tang *et al*., 2002). In 15-LOX2sv-c, the bold horizontal bar indicates the retained intron 12. 15-LOX2sv-d is identical to 15-LOX2 except that a 45-bp facultative intron in exon 9 (nt1302 – nt1346) is spliced out. 15-LOX2sv-e is identical to 15-LOX2sv-c except for the exon 9 being spliced out.

figure S2. *15-LOX2 expression in human prostate correlates with age*. Archival human prostate tissue sections of various ages (left) were processed for 15-LOX2 (A, C, E, and G) or PSA staining (B, D, F, and H). Original magnifications: x100 except H (x50).

Age (yr)	15-LOX2	PSA
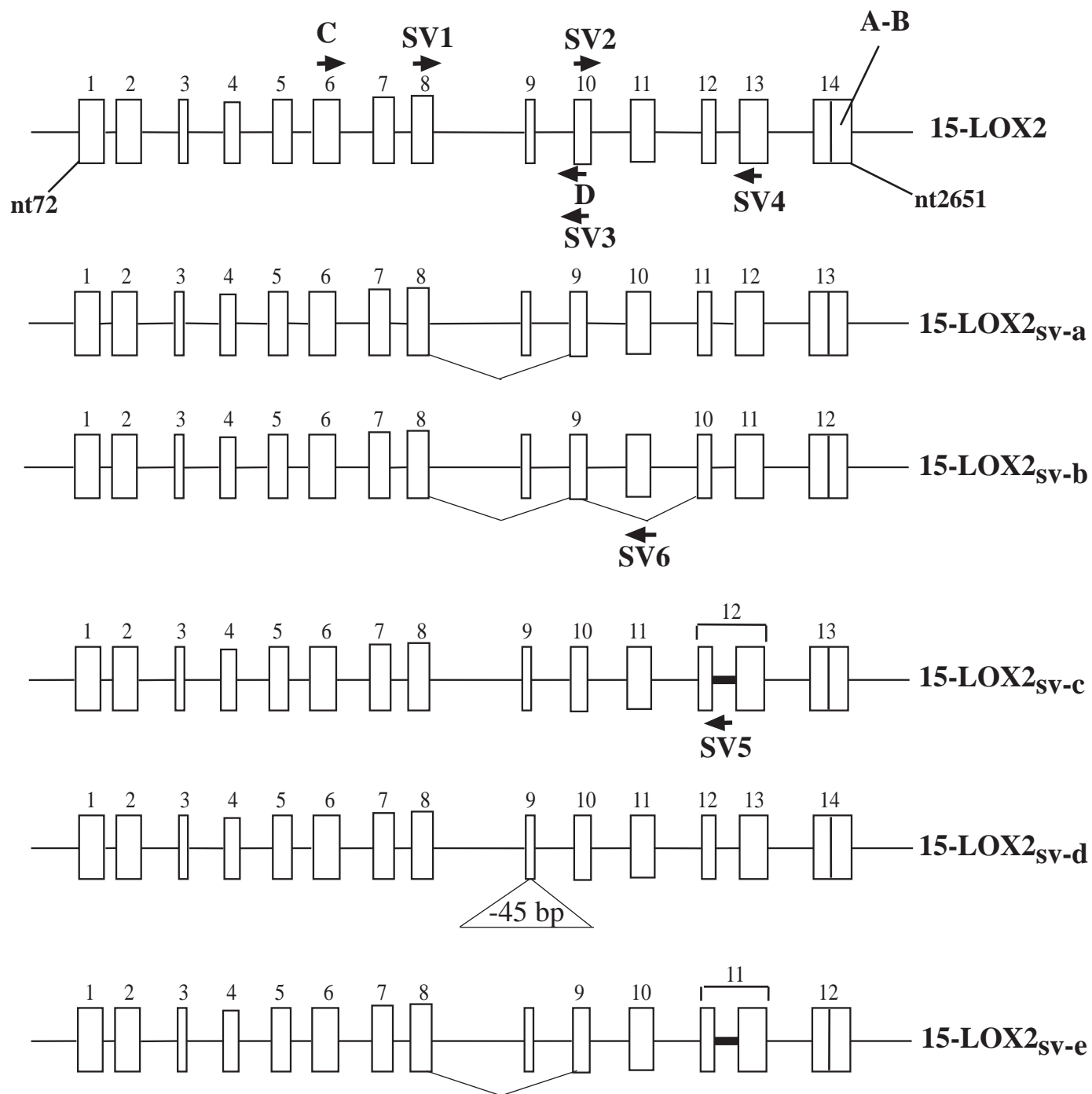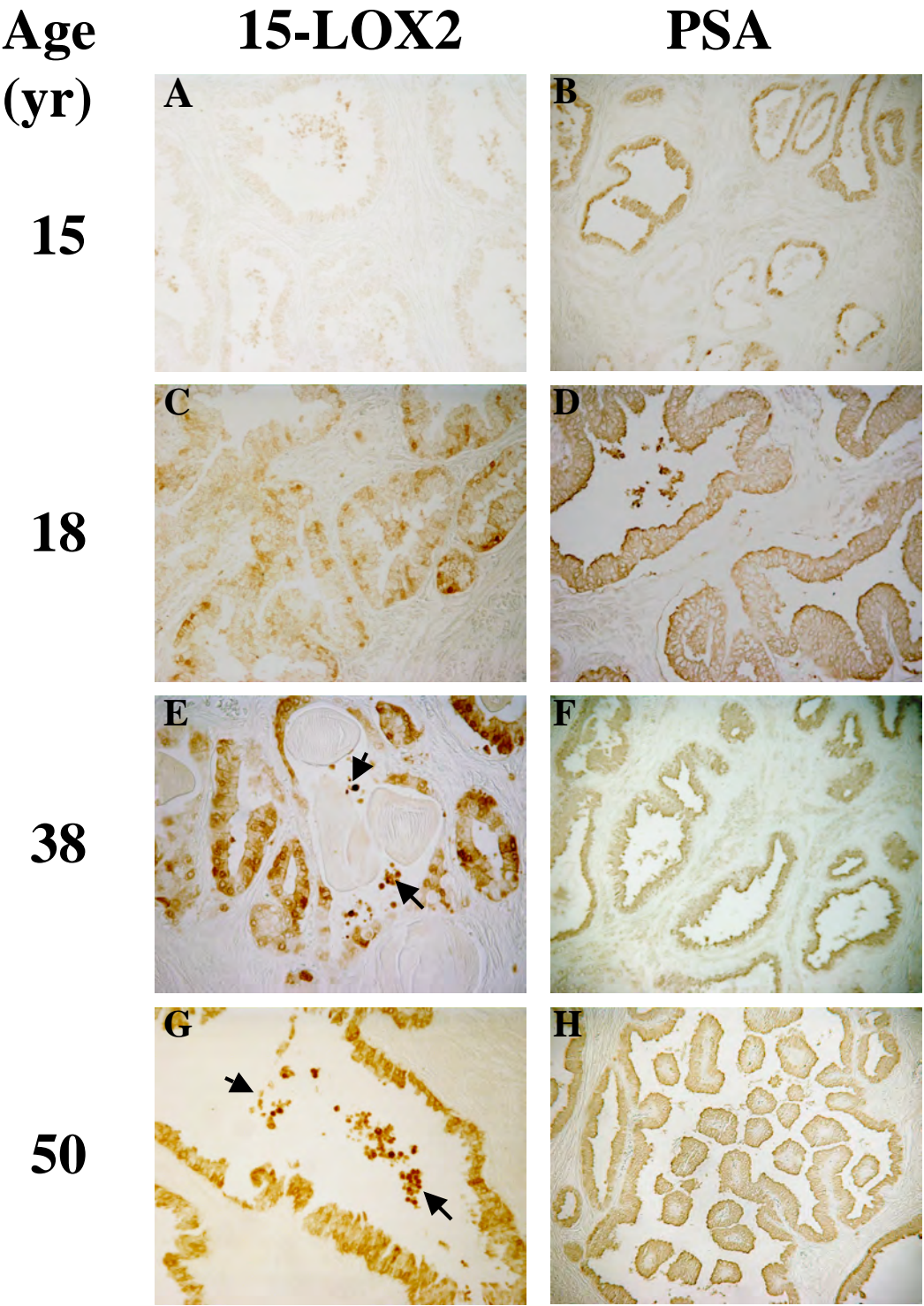
15-lipoxygenase 2 (15-LOX2) is a functional tumor suppressor that regulates human prostate epithelial cell differentiation, senescence, and growth (size)

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Running title: 15-LOX2 as a functional prostate tumor suppressor

Key words: 15-lipoxygenase 2, prostate epithelial cells, prostate cancer, cell senescence, stem cells, differentiation, cell growth

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Abstract

15-Lipoxygenase 2 (15-LOX2) is the major mammalian lipoxygenase expressed in normal human adult prostate and its expression is decreased or lost in high-grade prostate intraepithelial neoplasia (HGPIN) and prostate cancer (PCa). Our recent work has demonstrated that **1)** 15-LOX2 has multiple alternatively spliced isoforms and is a negative cell-cycle regulator in normal human prostate (NHP) epithelial cells; **2)** 15-LOX2 in NHP cells is positively regulated by Sp1 and negatively regulated by Sp3; **3)** 15-LOX2 in NHP cells may be partially involved in cell differentiation; **4)** 15-LOX2 is cell-autonomously upregulated in cultured NHP cells and its induction is associated with NHP cell senescence; and **5)** 15-LOX2 is a functional prostate tumor suppressor. Here we summarize these new findings to provide a concise view of the potential biological functions of 15-LOX2 in NHP cells and of its deregulation in PCa development.

15-Lipoxygenase 2 (15-LOX2) is one of the recently identified mammalian lipoxygenases (1). It shows the highest homology to murine 8-LOX and mainly metabolizes arachidonic acid (AA) to 15(S)-hydroxyeicosatetraenoic acid [15(S)-HETE] (1,2). In adult human, 15-LOX2 shows tissue-restricted expression pattern, with its protein expressed primarily in prostate followed by the lung, skin, and cornea (1) although its mRNA can be detected in some other tissues (2). The biological functions of 15-LOX2 in prostate and the other tissues remain unclear. 15-LOX2 expression and activity are decreased in high-grade prostate intraepithelial neoplasia (HGPIN) and prostate cancer (PCa) (3,4). 15-LOX2 expression is also downregulated in benign and neoplastic sebaceous glands (5), esophageal cancer (6), and lung cancer (7). These latter findings implicate abnormal 15-LOX2 regulation/expression in tumor development.

Potential Biological Functions of 15-LOX2 in Normal Human Prostate (NHP) Epithelial Cells

Since 15-LOX2 is normally expressed mainly in adult prostate, we decide to use the NHP cells as a model to study and understand the potential physiological functions of this molecule in the prostate. Human prostatic glands consist of two major epithelial cell types: basal and secretory (luminal). Basal cells express cytokeratin (CK) 5 and 14 whereas luminal cells express CK8 and 18, AR (androgen receptor), PSA (prostate-specific antigen), PAP (prostatic acid phosphatase), and CD57 (reviewed in 8). Evidence exists that the basal-cell compartment harbors prostate stem/progenitor cells whereas the luminal cells mostly represent terminally differentiated cells that perform secretory functions (8). 15-LOX2 is expressed exclusively in the luminal cell layer (3,9; also see Fig. 4A-C), suggesting that one of the biological functions of 15-

LOX2 in human prostate may be to induce and maintain the differentiated phenotype of the NHP cells.

A. 15-LOX2 has multiple alternatively spliced isoforms

During our work on 15-LOX2, we observed that 15-LOX2 has at least 6 different splice variants, which we have named as 15-LOX2sv-a to 15-LOX2sv-f (Fig. 1) (9,10). These splice variants have spliced out some key segments within the protein or the C-terminal His residue, both of which are important for the catalytic activity of the enzyme. Therefore, they are predicted to lack or have much reduced AA-metabolizing activities. Indeed, when we transfected 15-LOX2sv-a to 15-LOX2sv-c into PCa cells that do not express endogenous 15-LOX2 proteins, little 15(S)-HETE was detected (11; unpublished observations). These observations raise an intriguing question: if these splice variants do not have appreciable AA-metabolizing activities, why are they expressed? One possibility is that they possess biological functions unrelated to AA metabolism (i.e., they can still metabolize other lipid substrates; 2). Another possibility is that they have biological functions that are completely independent of lipid metabolism (see below).

A very interesting facet about these splice variants is that, just like the wild-type, full-length 15-LOX2, their expression is also lost/downregulated in PCa cells (9; also see 6A) but its expression accumulates in senescing NHP cells (10). This would suggest that these splice variants functionally may behave very similarly as the parental 15-LOX2. In support, in all of our gain-of-function experiments we have observed very similar biological activities (e.g., with respect to inhibiting cell-cycle progression and tumor development or inducing cell senescence) between 15-LOX2 and 15-LOX2sv-a to 15-LOX2sv-c (9-11). On the other hand, unlike 15-

LOX2, the 15-LOX2 splice splice variants are largely excluded from cell nucleus (11), suggesting that they likely also possess distinct biological functions.

B. 15-LOX2 expression in cultured NHP cells is cell-autonomously induced and is positively regulated by Sp1 and negatively by Sp3

Remarkably, when primary NHP cells are cultured in serum and androgen-free conditions containing one main survival factor insulin and one main mitogen EGF, they cell-autonomously upregulate the mRNA expression of 15-LOX2 as well as all its splice variants (10). The upregulation of 15-LOX2 mRNA leads to increased 15-LOX2 protein expression and 15(S)-HETE production in serially cultured NHP cells (12). What transcription factor(s) might be responsible for this cell-autonomous induction of 15-LOX2 in cultured NHP cells? We find that 1) several Sp1 sites in the proximal promoter region of the 15-LOX2 gene are important *cis* elements regulating the15-LOX2 promoter activity; 2) Sp1 and Sp3 are two major GC-binding *trans* factors regulating 15-LOX2 gene expression with Sp1 being a positive and Sp3 a negative regulator; and 3) multiple complexes form on the GC-rich regions and Sp1 and Sp3 exist in some of these complexes (12).

The 15-LOX2 promoter is TATAA-less (12), consistent with its constitutive expression in adult NHP cells in vivo. Sp1 sites including GC-boxes and CACCC-boxes (also called GT boxes) have been shown to be responsible for recruiting TATA-binding protein and fixing the TSS (transcription start site) on TATAA-less promoters. *In silico* analysis identifies 4 potential Sp1 sites in the proximal promotor region of 15-LOX2 gene (12). Initial evidence that these Sp1 sites may play a role in regulating 15-LOX2 comes from deletion analysis. More definitive evidence comes from site-specific mutagenesis, which suggests that GC box 2 and/or 3 may be

5

more important than the GC box 1/CACCC box, as well as from EMSA competition experiments, which suggest that GC box 2 is the primary Sp1 site that multiple transcription factors bind (12). In the Sp subfamily, Sp3 is the only protein that can either positively or negatively modulate the Sp1-dependent gene expression. Our subsequent experiments demonstrate that Sp3 dose-dependently inhibits the Sp1-activated 15-LOX2 promoter activity as well as endogenous 15-LOX2 expression in NHP cells (12). Together, these results establish that Sp1 and Sp3 are biologically relevant and essential regulators of the 15-LOX2 gene expression in cultured NHP cells with Sp1 being an activator and Sp3 an inhibitor via antagonizing Sp1 activity (12).

C. *15-LOX2 expression in NHP cells is not directly regulated by androgen/AR*

Since 15-LOX2 is normally expressed in the differentiated luminal cells, which are usually regulated by the androgen/AR signaling pathway, it is reasonable to think that 15-LOX2 expression might be also regulated by androgen. Consistent with this thinking, NHP cells in culture gradually upregulate the AR mRNA expression although the AR protein is never detectable (11,12). However, several pieces of evidence suggest that the androgen/AR pathway does not directly regulate 15-LOX2 expression in NHP cells. *First*, there is no authentic ARE (androgen-responsive element) in the 16 kb upstream sequence of 15-LOX2 promoter (12). *Second*, although there is a partially matched ARE at ~-1.4 kb location, a 4 kb fragment containing this partially-matched ARE does not possess testosterone-inducible promoter activity (12). *Third*, exogenous androgen together with enforced expression of AR does not induce endogenous 15-LOX2 gene expression in young NHP cells although they can readily activate a 15-LOX2 transgene driven by the probasin promoter (12). *Fourth*, we have observed that 15-

LOX2 expression is cell-autonomously induced in NHP cells cultured in the absence of androgen or serum (10). *Fifth*, 15-LOX2 gene expression in human prostates comes after PSA expression (10), probably as a consequence of prostate differentiation. *Finally*, that androgen does not directly regulate 15-LOX2 gene expression is also consistent with clinical data that PCa tend to have heightened AR activity leading to increased PSA production but they downregulate or lose 15-LOX2 expression (3,4,9).

D. 15-LOX2 expression in NHP cells may be related to E-cadherin-mediated cell differentiation

The excusive expression of 15-LOX2 expression in luminal prostatic cells suggests that the molecule must be playing biological functions related to cell differentiation and secretion. Consistent with this postulate, NHP cells in culture gradually upregulate both AR mRNA and 15-LOX2 mRNA and protein and the cells morphologically manifest bigger and flat phenotype (10), suggesting that the cells are probably undergoing partial differentiation. It is still not clear what molecules might represent the 'master' differentiation regulators in NHP cell differentiation. We examined a panel of NHP and PCa cells for the expression of 15-LOX2 and 3 differentiation-related molecules, i.e., PPARα (peroxisome proliferators activating receptor α), PPARγ, and E-cadherin. As shown in Fig. 2A, the full-length 15-LOX2 protein is detected in all 5 primary strains of NHP cells but not in any of the 9 PCa cell lines, consistent with our earlier observations (9). Interestingly, in this experiment, PCa cells do express some lower M.W. 15-LOX2 splice variants (Fig. 2A). Different from 15-LOX2, both PPARα and PPARγ are detected in all cells examined (Fig. 2A). The E-cadherin expression pattern in these cells is very revealing. In NHP cells, in addition to the full-length, 120 kD E-cadherin, two truncated E-

cadherin proteins designated as E-cad^{97} and E-cad^{80}, both of which have previously been reported (13-15), are also observed (Fig. 2A). Furthermore, we have also observed a prominent doublet of E-cad$^{58/60}$ in NHP cells (Fig. 2A), which most likely represent novel E-caderin isoforms/truncation products. PCa cells show very different E-cadherin expression patterns than in NHP cells – most do not express the E-cad$^{58/60}$, 4 of them (PPC-1, Du145, JCA-1, and Tsu-Pr) completely lack all E-caderin expression, and the other 5 (MDA 2b, LNCaP, LNCaP-C4-2, LNCaP-C5, PC3) express the full-length E-cadherin, different levels of 3 truncation products (i..e., E-cad^{97}, E-cad^{80}, and E-cad$^{58/60}$), and, in addition, a new E-cadherin protein E-cad^{45} (Fig. 2A). Overall, it appears that PCa cells tend to lose the expression of both 15-LOX2 and E-cadherin (Fig. 2A), thus suggesting that these two molecules might be somehow linked.

E-cadherin is a critical cell-cell adhesion molecule that maintains the epithelial integrity and cytodifferentiation. It is well-documented that E-cadherin expression is often lost or abnormally regulated in multiple epithelial cancer cells, including PCa, possibly due to promoter hypermethylation (16-18) and some other mechanisms such as protein truncations mentioned above (13-15). Interestingly, in our subcellular distribution studies of 15-LOX2, we have observed that 15-LOX2, in addition to its prominent cytoplasmic and nuclear localizations, is also expressed along cytoskeleton as well as cell-cell borders where it colocalizes with E-caderin in NHP cells (11). In addition, elevated levels of calcium in the culture medium further promotes 15-LOX2 localization to the cell-cell junctures (Fig. 2Bb) where E-cadherin is normally concentrated. Although it is not yet clear whether 15-LOX2 and E-caderin physically interact, these observations (11; Fig. 2) raise an intriguing possibility that the two proteins might be interacting in some way to help induce and maintain the differentiated phenotype of the NHP epithelial cells. In PCa, both molecules tend to be deregulated and lost.

E. 15-LOX2 expression in NHP cells is involved in cell senescence

NHP cells, like most somatic human cells, not only undergo functional differentiation but also become chronologically senescent in vivo. Indeed, senescence-associated β-galactosidase (SA-βgal) positive, senescent NHP cells have been detected in adult human prostate (19,20). NHP cells cultured in serum/androgen-free medium also undergo senescence after 20-26 population doublings (PDs) (10). Significantly, before the NHP cells in culture approach the end of their prolferative lifespan and become senescent, they cell-autonomously upregulate the mRNA and protein levels of 15-LOX2 and all its splice variants (10). The upregulated 15-LOX2 is enzymatically active as it leads to increasing production of 15(S)-HETE in the culture medium (10). One piece of evidence that the cell-autonomously induced 15-LOX2 and its splice variants are causally involved in NHP cell senescence is that 15(S)-HETE, at 25 μM, could induce an a senescence-like phenotype in NHP as well as in PCa cells – cells treated for as short as 72 h showed typical enlarged, flattened, and immotile morphology (Fig. 3) and the cells are generally arrested in cell cycle (Table 1) (9-11).

The most convincing evidence that implicates 15-LOX2 accumulation in NHP cell senescence is through the gain-of-function experiments, in which we infected the young NHP cells with retroviral vectors encoding 15-LOX2, 15-LOX2sv-a, or 15-LOX2sv-b. The results reveal that enforced expression of 15-LOX2 or its splice variants is sufficient to drive a small fraction (up to ~10%) of the young NHP cells to cell-cycle arrest and senescence (10). These observations suggest that 15-LOX2 may represent one of the regulators of NHP cell senescence. The key unanswered question is whether prevention of cell-autonomous induction of 15-LOX2 in NHP cells will be sufficient to delay or prevent NHP cell senescence.

F. 15-LOX2 might be involved in regulating cell growth (or size), a common denominator for both differentiation and senescence

Both functionally differentiated and chronologically senescent cells are significantly larger in size than their corresponding proliferating young progenitor cells, due to continued cell growth (i.e., size increase) without undergoing cell cytokinesis and cell division. It is interesting to note that 15-LOX2 expression in NHP cells is invariably correlated, inversely, with cell size, i.e., all 15-LOX2-expressing NHP cells are significantly (frequently >10 times) larger than the 15-LOX2-negative NHP cells (9,10). As NHP cells in culture also undergo partial differentiation (i.e., increasing AR mRNA expression) and senescence, these observations (9,10) suggest that 15-LOX2 might actually be regulating cell growth, a common denominator of both cell differentiation and cell senescence. We are currently exploring this possibility in a transgenic animal model.

15-LOX2 expression and PCa development: Evidence for a functional tumor suppressor

A. 15-LOX2 expression is lost in all immortalized and tumorigenic prostate cells

Early evidence that links 15-LOX2 and tumor development is its downregulation or loss of expression in PCa (3,4) as well as in several other malignancies including neoplastic sebaceous glands (5), esophageal cancer (6), and lung cancer (7). In normal prostatic glands, 15-LOX2 is expressed in either scattered cells or in contiguous patches of luminal cells in the cytoplasm, nucleus, and cell-cell borders (Fig. 4A-C) (9,11). Remarkably, in PIN or PIN-like precursor lesions where epithelial cells have lost the single-layer organization and piled up, 15-LOX2

expression is invariably lost (Fig. 4D-F), suggesting that loss of 15-LOX2 expression may represent an early event in PCa development.

Loss of 15-LOX2 expression is even more dramatic in cultured immortalized and tumorigenic prostate cells - in >20 immortalized prostate epithelial cells and established prostate cancer cells, we could not detect 15-LOX2 protein expression (Fig. 2A; Fig. 4G) (9). For example, 15-LOX2 is detected in prostate epithelial cells prepared from two patients, RC-176N and RC-81N, but not in corresponding, preimmortalized RC176T and RC-81T cells (Fig. 4G), suggesting that 15-LOX2 expression was lost in vivo. Immortalization with either hTERT (RC-176N hTERT) or E6/E7 (RC-81N E6/E7) rendered the epithelial cells to lose 15-LOX2 expression (Fig. 4G). Similarly, immortalization with other means such as HPV-18 and SV40 also caused the loss of 15-LOX2 (Fig. 4G). Also, 15-LOX2 is not expressed in immortalized 267B1 cells or the transformed cells (267B1/x-ray) (Fig. 4G). The fact that 15-LOX2 expression is lost accompanying the acquisition of prostate epithelial cell immortality is fully consistent with the idea that 15-LOX2 is involved in NHP cell growth arrest and senescence (10).

B. Loss of 15-LOX2 expression in PCa cells does not result from gene mutations, DNA hypermethylation, abnormal Sp1 expression, or loss/downregulation of KLF6

How is 15-LOX2 expression lost in immortalized prostate epithelial cells and PCa cells? We sequenced the coding regions of 15-LOX2 from several PCa cell lines (PC3, LNCaP, Du145 and PPC-1) and did not find any genomic mutations (9), suggesting that loss of 15-LOX2 expression is unlikely caused by gene mutations. Treatment of PCa cells with inhibitors of DNA methyltransferases 5-aza-deoxycytidine and/or trichostatin A (TSA) also fail to upregulate 15-

LOX2 protein expression in PCa cells (9), suggesting that shutting down of 15-LOX2 expression is not caused by promoter or general gene hypermethylation.

Loss of 15-LOX2 protein expression in PCa cells occurs at the transcriptional level as evidenced by both dampened 15-LOX2 gene promoter/enhancer activities (Fig. 5A-C) as well as lack of 15-LOX2 mRNA expression (9). Since 15-LOX2 gene transcription in NHP cells is positively regulated by the Sp1 transcription factor (12), is it possible that 15-LOX2 gene silencing in PCa cells is caused by deregulated Sp1 expression/functions? Somewhat surprisingly, PCa cells actually express higher amounts of Sp1 protein than NHP cells (12), suggesting that loss of 15-LOX2 expression in PCa cells is unlikely due to decreased Sp1 protein expression. In support, enforced expression of Sp1 protein in PCa cells does not appreciably increase the 15-LOX2 promoter activities (Fig. 5D).

Recently, another Sp1 family protein, KLF6, has been proposed as a candidate prostate tumor suppressor due to genomic mutations (21-24). Since KLF6 shows very similar binding profiles to Sp1 on target promoters, we tested the hypothesis that loss of 15-LOX2 expression in PCa cells might be related to lack of KLF6 expression. As shown in Fig. 6A, KLF6 expression was detected in all 4 NHP cell strains but only in 1 (i.e., LNCaP) of the 4 PCa cell lines, consistent with the idea that KLF6 expression is downregulated/lost in PCa cells (21-24). However, enforced expression of either wild-type KLF6 or a KLF6 mutant (i.e., KLF6M) (21) in PC3 cells (Fig. 6B) failed to enhance the 15-LOX2 promoter activity (Fig. 6C) or restore 15-LOX2 protein expression (Fig. 6B).

These observations together suggest that the transcriptional silencing of 15-LOX2 expression in PCa cells is likely mediated by complex molecular mechanisms involving abnormal transcription factor recruitment and chromatin remodeling.

C. 15-LOX2 is a functional prostate tumor suppressor

What evidence supports our claim that 15-LOX2 represents a *functional* prostate tumor suppressor? *First*, 15-LOX2 expression is downregulated or lost in PCa as well as in several other cancers (3-7), consistent with it being a potential tumor suppressor. *Second*, on the other hand, there are no somatic mutations in the 15-LOX2 coding regions in PCa cells (9), thus excluding 15-LOX2 as a conventional tumor suppressor. *Third*, 15-LOX2 expression in NHP cells is correlated with cell-cycle arrest and senescence (10). Since cell senescence program is one of the most powerful tumor suppressor mechanisms, 15-LOX2 accumulation in NHP cells likely constitutes an inhibitory mechanism that limits uncontrolled cell proliferation associated with tumor development. *Fourth*, enforced expression of 15-LOX2 in both young NHP (10) and PCa (9,11) cells induce cell-cycle arrest and a senescence-like phenotype, consistent with 15-LOX2 being a potential tumor suppressor. *Finally*, and most importantly, enforced expression of 15-LOX2 in PCa cells inhibit tumor development in vivo (11).

Functional relationship between 15-LOX2 and its AA metabolite, 15(S)-HETE

15-LOX2 has been conventionally considered an enzyme that mainly metabolizes the phospholipids, in particular, AA. In other words, many of the purported functions of 15-LOX2 are thought to be mediated through its major metabolite 15(S)-HETE. Indeed, we have already shown that 15(S)-HETE can induce a senescence-like phenotype in NHP and PCa cells (Fig. 4). Exogenous 15(S)-HETE also inhibits NHP and PCa cell proliferation (Table 1; 9,11) as well as PCa cell transmigration across the Boyden chambers (Table 2). However, the biological effects of 15(S)-HETE are noticeable generally at >10 – 25 µM, raising the question of physiological relevancy of these in vitro observations, i.e., whether the in vivo biological activities of 15-

LOX2 are actually mediated by 15(S)-HETE considering the difficulty in achieving such high concentrations of 15(S)-HETE in the cells.

Furthermore, the fact that 15-LOX2 is clearly expressed at multiple distinct subcellular locations (cytoplasm, nucleus, cell-cell borders, cytokeleton, and plasma membrane; 11) suggests that 15-LOX2 likely possesses multiple biological functions, some of which may not necessarily be dependent on AA or even lipid metabolism. The best evidence supporting this latter supposition is that in all of our gain-of-function experiments using 15-LOX2sv-b, which does not produce 15(S)-HETE at all, we have observed nearly identical biological activities (e..g, inhibition of cell-cycle progression and proliferation, induction of a senescence-like phenotype, and inhibition of tumor development in vivo; 9-11) to those achieved with 15-LOX2. These results clearly indicate that the biological activities of 15-LOX2 do not necessarily have to be mediated through its enzymatic activity to generate 15(S)-HETE. One hypothetical scenario we proposed earlier (11) is that 15-LOX2 may possess both AA metabolism-dependent and AA metabolism-independent mechanisms of action. For instance, through 15-LOX2 expression in the nucleus and concentrated 15(S)-HETE production in the organelle, 15(S)-HETE might be able to function as a potential PPARγ ligand to activate the downstream targets. Through its extra-nuclear expression, in particular, through the actions of 15-LOX2 splice variants that are largely excluded from the nucleus, 15-LOX2 and its splice variants may also carry out AA metabolism-independent functions (11). Together, these two mechanisms may work in concert to exert permanent cell-cycle arrest in NHP cells thus helping cell differentiation and senescence. Loss of expression of 15-LOX2 and its splice variants will undoubtedly lead to uncontrolled cell proliferation and loss of differentiation/senescence and thus contribute to PCa development.

REFERENCES

1. Brash, A.R., Boeglin, W.E., and Chang, M.S. Proc. Natl. Acad. Sci. USA 94:6148, 1997.

2. Kilty, I., Alison, L., and Vickers, P.J. Eur. J. Biochem. 266:83, 1999.

3. Shappell, S.B., Boeglin, W.E., Olson, S.J., Kasper, S., and Brash, AR. Am. J. Pathol. 155:235, 1999.

4. Shappell, S.B., Manning, S., Boeglin, W.E., et al., Neoplasia 3:287, 2001.

5. Shappell, S.B., Keeney, D.S., Zhang, J., Page, R., Olson, S.J., and Brash, A.R. J. Invest. Dermatol. 117:36, 2001.

6. Xu, X.C., Shappell, S.B., Liang, Z., et al., Neoplasia 5:121, 2003.

7. Gonzalez, A.L., Roberts, R.L., Massion, P.P., Olson, S.J., Shyr, Y., and Shappell, S.B. Hum. Pathol. 35: 840, 2004.

8. Tang, D.G., Patrawala L, Calhoun T, et al. Mol. Carcinog. 2006, *in press*.

9. Tang, S., Bhatia, B., Maldonado, C., et al., J. Biol. Chem. 277: 16189, 2002.

10. Bhatia, B., Tang, S., Yang, P., et al., Oncogene 24: 3583, 2005.

11. Bhatia, B., Maldonado, C.J., Tang, S., et al., J. Biol. Chem. 278: 25091, 2003.

12. Tang, S., Bhatia, B., Zhou, J-J., et al., Oncogene 23: 6942, 2004.

13. Rashid, M.G., Sanda, M.G., Vallorosi, C.J., Rios-Doria, J., Rubin, M.A., and Day, M.L. Cancer Res. 61: 489, 2001.

14. Rios-Doria, J., Day, K.C., Kuefer, R., et al., J Biol Chem. 278: 1372, 2003.

15. Kuefer, R., Hofer, M.D., Gschwend, J.E, et al., Clin Cancer Res. 9: 6447, 2003.

16. Li, L.C., Zhao, H., Nakajima, K., et al., J. Urol. 166: 705, 2001.

17. Kallakury, B.V., Sheehan, C.E., Winn-Deen, E., et al., Cancer 92: 2786, 2001.

18. Woodson, K., Hayes, R., Wideroff, L., Villaruz, L., and Tangrea, J. Prostate 55: 199, 2003.

19. Castro, P., Giri, D., Lamb, D., and Ittman, M. Prostate 55: 30, 2003.

20. Choi, J., Shendrik, I., Peacocke, M., et al., Urology 56: 160, 2000.

21. Narla, G., Heath, K.E., Reeves, H.L., et al., Science 294: 2563, 2001.

22. Chen, C., Hyytinen, E.R., Sun, X., et al., Am. J. Pathol. 162: 1349, 2003.

23. Narla, G., Difeo, A., Reeves, H.L, et al., Cancer Res. 65: 1213, 2005.

24. Narla, G., DiFeo, A., Yao, S., et al., Cancer Res. 65: 5761, 2005.

Acknowledgement

FIGURE LEGENDS

Figure 1. *Schematic of 15-LOX2 splicing isoforms (15-LOX2sv)*. The numbering system is based on Brash *et al*. (1) with the translational ATG starting at nucleotide (nt) 72 in exon 1. The structures for 15-LOX2sv-a to 15-LOX2sv-c have been described in detail (9). In 15-LOX2sv-c, the bold horizontal bar indicates the retained intron 12. 15-LOX2sv-d is identical to 15-LOX2 except that a 45-bp facultative intron in exon 9 (nt1302 – nt1346) is spliced out. 15-LOX2sv-e is identical to 15-LOX2sv-c except for exon 9 being spliced out. 15-LOX2sv-f is identical to 15-LOX2sv-e except for exon 10 being spliced out. Individual PCR primers (i.e., A-D and SV1-SV6) used to differentially amplify these splice variants from NHP and/or PCa cells are indicated. For experimental details, see ref. 9 and 10.

Figure 2. *Correlation between 15-LOX2 and E-cadherin expression in NHP and PCa cells*. (**A**) Western blot analysis of the molecules indicated (100 μg/lane of total cell lysates). NHP cells are primary cell strains and all other cells are established PCa cell lines (9). (**B**) Representative microphotographs showing NHP6 cells either cultured in regular PrEBM culture medium containing 0.1 mM calcium (**a**) or in the same medium containing 1 mM calcium (**b**). Note that in a, 15-LOX2 is detected mostly in the cytoplasm and nucleus whereas in b much of 15-LOX2 becomes concentrated towards cell-cell borders. Original magnifications, x400.

Figure 3. *Induction of a senescence-like phenotype by 15(S)-HETE in both NHP and PCa cells*. NHP2, TP1 (a primary PCa cell strain; 9), and PC3 cells were treated with either the vehicle

(control) or 15(S)-HETE. Images were taken 72 h post treatment. Original magnifications: x200.

Figure 4. *Loss of 15-LOX2 expression in PIN-like lesions in vivo and in immortalized prostate epithelial cells in vitro*. (**A-F**) IHC analysis of 15-LOX2 expression in normal prostatic glands (A-C) or in PIN-like lesions (D-F). Note that in normal glands, 15-LOX2 is distinctively expressed in cell nucleus, cytoplasm, and cell-cell borders in either scattered cells (B and C) or in contiguous layers (A). In contrast, in PIN-like lesions (D-F) where epithelial cells have lost the single-cell layer organization and piled up, 15-LOX2 expression is lost (demarcated by dotted lines). In D and F, arrows point to 15-LOX2 expression in the neighboring normal glands. Original magnifications, x400. (**G**) Loss of 15-LOX2 expression in immortalized prostate epithelial cells. Western blot analysis of 15-LOX2 in immortalized cells (100 μg/lane total cell lysates). See Text for details.

Figure 5. *15-LOX2 gene promoter activity is suppressed in PCa cells*. (**A-B**) Luciferase reporter assays (n = 5) were performed using NHP6 cells (passage 5-7) as well as PCa cells (TP1, LNCaP, PPC-1, and PC3) as previously described (12). The relative promoter activities (RLU) for either the P4 – P8 (-471/+80) (A) or the P7 – P8 (–1116/+80) fragment (12) were presented. (**C**) The potential enhancer activity of 15-LOX2 promoter (i.e., P7-P8 fragment) is also downregulated in PCa cells. In this set of experiments, the P7-P8 15-LOX2 promoter fragment was cloned into a pGL3.enhancer construct that contains endogenous minimal SV40 promoter and then luciferase experiments were similarly performed 72 h after

transfection. (**D**) Exogenous Sp1 protein expression does not significantly increase 15-LOX2 promoter activity in PCa cells.

Figure 6. *Decreased/lack of KLF6 expression in PCa cells but enforced KLF6 expression fails to restore 15-LOX2 expression.* (**A**) Western blot analysis of 15-LOX2 and KLF6 in NHP and PCa cells (100 µg/lane total cell lysates). (**B**) PC3 cells were either untransfected (UT), or transfected with empty plasmid (CTL) or with a plasmid encoding KLF6 or a mutant KLF6 (KLF6M). 72 h after transfection, cells were harvested for Western blot analysis of KLF6, 15-LOX2, or actin. NHP6 cells were used as positive control for 15-LOX2. (**C**) PC3 cells were co-transfected with pGL3-basic plus the P6 –P8 15-LOX2 promoter fragment (12) or triple-tranfected with pGL3-basic plus the P6 –P8 15-LOX2 promoter fragment plus various KLF6-related constructs. Luciferase activities were measured 72 h after transfection (n = 5) and are expressed as the relative promoter activities (12).

Table 1. Effect of 15(S)-HETE on prostate (cancer) cell proliferation and survival*

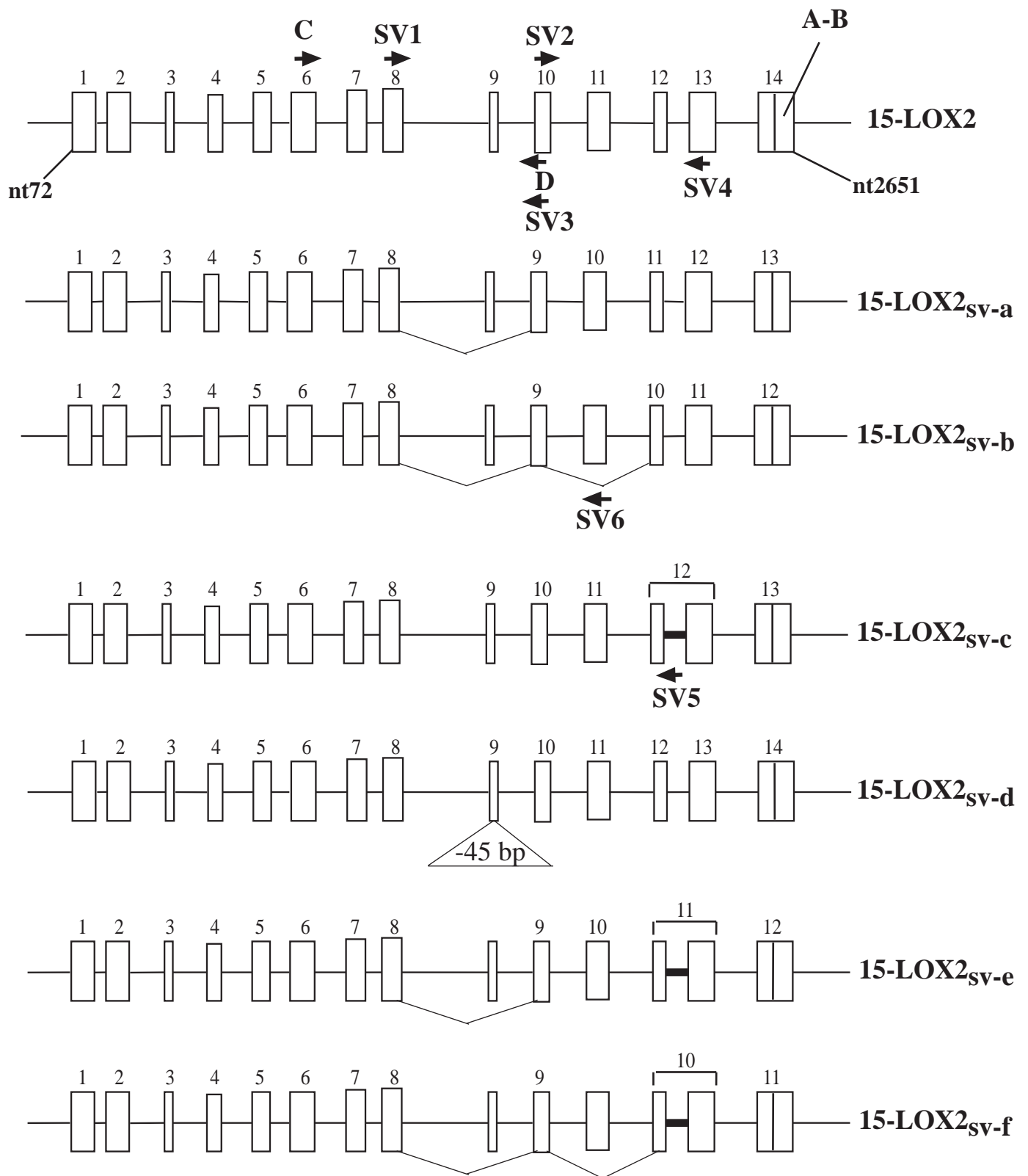| 15(S)-HETE (µM)	NHP2	LNCaP	PC3	Du145	NHP2	LNCaP	PC3	Du145
	(% of control)				(% of cells plated)			
0	100	100	100	100	375 ± 9	180 ± 20	160 ± 27	238 ± 22
1	102 ± 4	123 ± 20	86 ± 3	62 ± 11	388 ± 12	221 ± 30	138 ± 5	147 ± 26
10	93 ± 6	68 ± 9	63 ± 3	50 ± 4	342 ± 25	122 ± 15	101 ± 4	117 ± 9
25	64 ± 3	50 ± 3	49 ± 3	37 ± 3	247 ± 12	89 ± 6	79 ± 5	87 ± 8
50	46 ± 3	16 ± 5	34 ± 1	30 ± 4	176 ± 11	29 ± 10	54 ± 5	72 ± 9
75	30 ± 4	0	0	0	112 ± 15	0	0	0

*NHP2, LNCaP, PC3, and Du145 cells were plated in 24-well culture plates at $1x10^4$ cells/well. Next day, cells were treated with 15(S)-HETE. NHP2 cells were treated in their normal serum-free culture medium (PrEBM) supplemented with EGF, insulin, hydrocortisone, and bovine pituitary extract whereas LNCaP, PC3, and Du145 cells were treated in RPMI 1640 medium supplemented with 2% FBS (instead of 5% FBS in their normal culture medium to reduce 15(S)-HETE binding to serum proteins). 72 h later, cells were harvested and the number of live cells was determined using trypan blue dye exclusion assays. Each condition was run in quadruplicate and the results are expressed either as the mean % cell number [relative to vehicle (ethanol) control] ± SD or as the mean % of initially plated cell number. The experiment was repeated twice with comparable results.
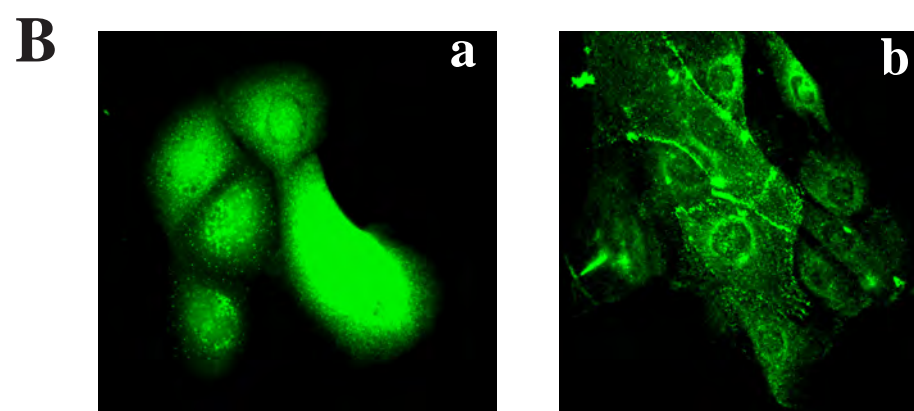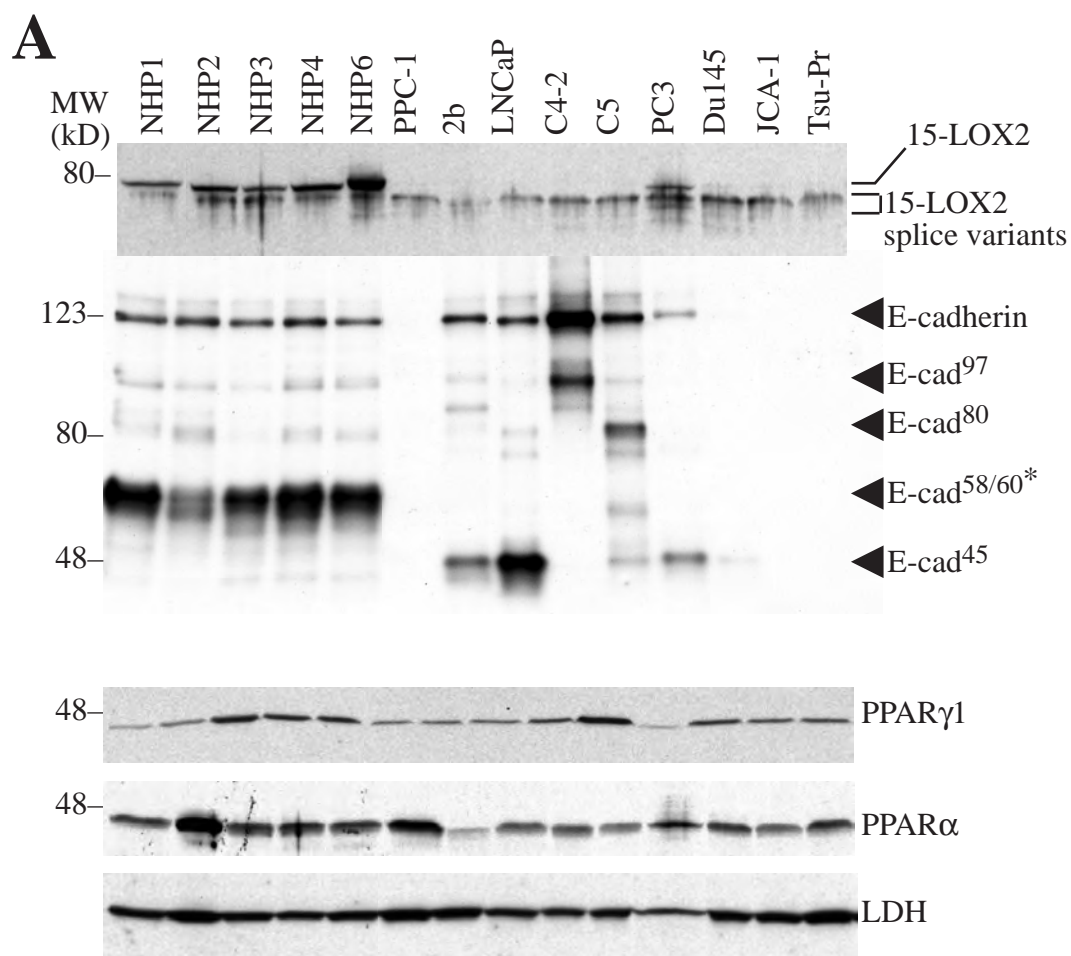
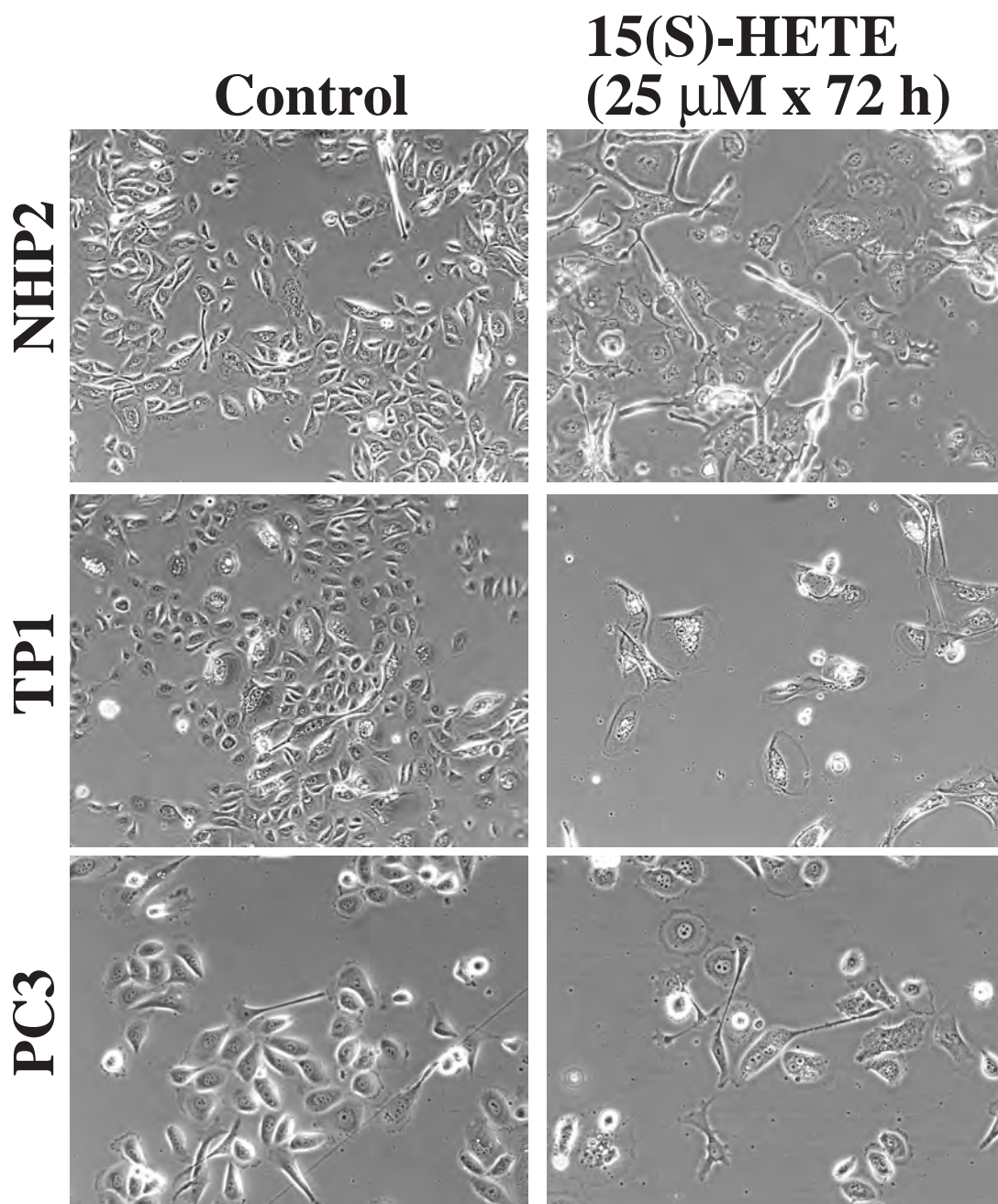**Table 2. Effect of 15(S)-HETE on prostate (cancer) transmigration (% of the total)***

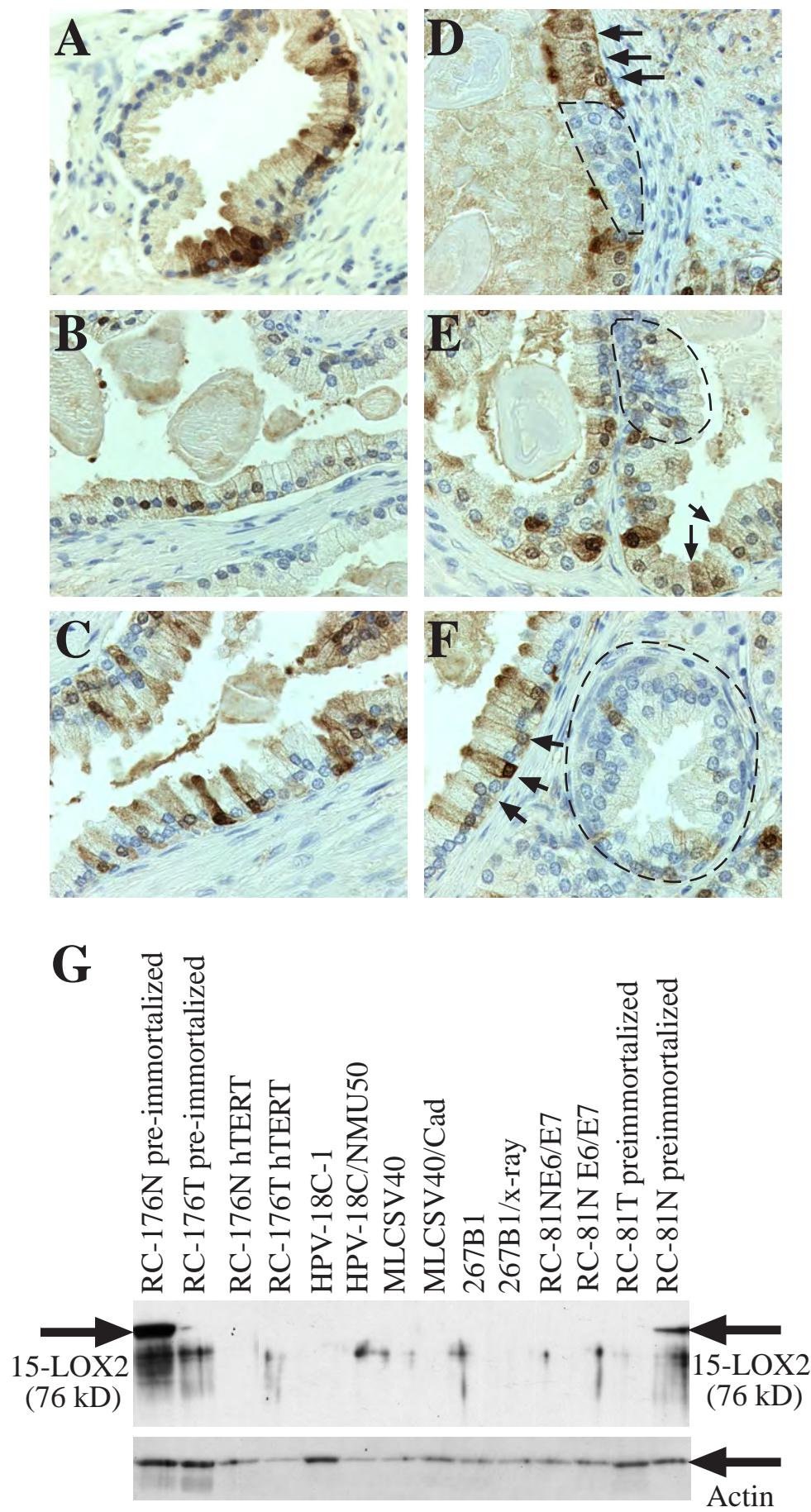| 15(S)-HETE (µM)	NHP2	LNCaP	Du145
0	0	75	80
5	0	N.D	8[#]
10	0	5.2[#]	4[#]
25	0	1.8[#]	2[#]

*NHP2, PC3, and Du145 cells were plated on 8 µm Boyden chamber membrane at $1x10^4$ cells/well in the absence or presence of 15(S)-HETE. Cells were treated as described in Table 1. 48 h later, cells on the top chamber were removed by a cotton swab and cells invaded (i.e., transmigrated) into the bottom side were counted after staining with Giemsa. The results are expressed as the % cells migrated across. Another independent experiment revealed comparable results. N.D., not determined.
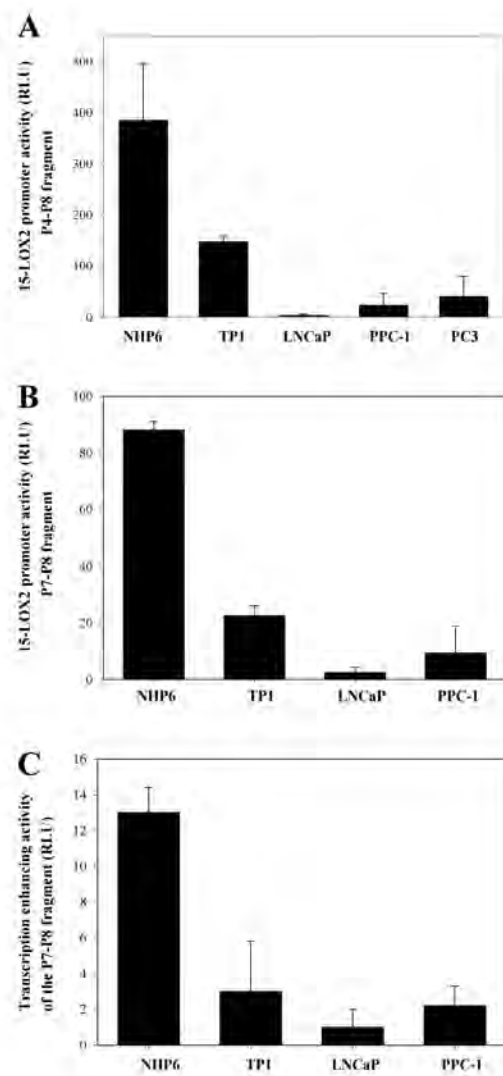*[#]$p<0.01$ (Student t-test).*

A

MW (kD)

NHP1 NHP2 NHP3 NHP4 NHP6 PPC-1 2b LNCaP C4-2 C5 PC3 Du145 JCA-1 Tsu-Pr

80–

15-LOX2
15-LOX2 splice variants

123– E-cadherin

E-cad^{97}

80– E-cad^{80}

E-cad$^{58/60*}$

48– E-cad^{45}

48– PPARγ1

48– PPARα

LDH

B

a

b

15-LOX2
(76 kD)

15-LOX2
(76 kD)

Actin

A

B

C